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**Investigating the role of T cells in the
progression of reflux oesophagitis to
Barrett's oesophagus and oesophageal
adenocarcinoma.**



**Trinity
College
Dublin**

The University of Dublin

A thesis submitted to Trinity College Dublin
for the degree of Doctor of philosophy

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Under the supervision of Dr. Joanne Lysaght

Department of Surgery

2016

Declaration

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Abstract

Oesophageal adenocarcinoma (OAC) is an inflammatory driven cancer that arises on a background of gastro-oesophageal reflux disease (GORD) and Barrett's oesophagus (BO). T cells have been implicated in the initiation of oesophagitis in GORD; however, their role in the progression from oesophagitis to BO and OAC has not been fully elucidated. The hypothesis of this thesis was that factors present in the local oesophageal microenvironment in oesophagitis, BO and OAC could alter T cell phenotype and function and ultimately drive disease progression. As such we carried out a novel and in-depth analysis of T cell profile in the GORD-BO-OAC sequence; examined the in-depth composition of the local oesophageal microenvironment, and assessed its impact of T cell profile and function, identifying potential immunotherapeutic targets.

Results supported findings of a number of phenotypic switches in cytokine profile in the progression from oesophagitis to BO and OAC. A pro-inflammatory profile was observed in the oesophagitis microenvironment with increased levels of IFN- γ and IL-2 demonstrated; the Barrett's microenvironment contained predominantly Th2 like cytokines including IL-10 and IL-6. Within the tumour microenvironment we demonstrated a mixed profile with higher levels of both pro- and anti-inflammatory cytokines. Factors present in the oesophageal microenvironment were found to significantly impact T cell cytokine profile and function, potentially driving Barrett's metaplasia. Furthermore, for the first time we identified increased IL-4 producing Th2 cells within Barrett's tissue and examined mechanisms for their accumulation in oesophageal tissue. This study identified a potential role for CCR3/CCR4 and CCL5, MIP1- α and CCL17 pathways in the recruitment of Th2 cells to BO and OAC tissue and demonstrated their potential for therapeutic intervention. Data from this thesis also identified a reduced proportion of activated T cells within OAC tissue and suggested that it was related to the lack of glucose in the oesophageal microenvironment. Finally we identified a novel mechanism for the Th2 polarisation observed in BO tissue, with glucose deprivation increasing IL-10 and IL-4 producing cells through alterations in T cell metabolism.

Overall this thesis suggests that secreted factors released from the tissue microenvironment may alter T cell phenotype and function, potentially playing a role in disease progression through novel mechanisms identified. We have identified potential novel therapeutic targets, which may hopefully improve understanding and treatment options for treating GORD, BO and OAC.

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Thesis outputs

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“The esophagitis to adenocarcinoma sequence; the role of inflammation”

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“Evaluating the role of T cells in the progression of Barrett's Oesophagus to Oesophageal adenocarcinoma”

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“Decreased infiltration and altered activation status of T cells occurs during the carcinogenic process from Barrett's Oesophagus to Oesophageal Adenocarcinoma”

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1. Chapter 1- General Introduction

1.1 The oesophagitis to adenocarcinoma sequence

The development of oesophageal adenocarcinoma (OAC) provides an excellent model of an inflammatory driven neoplastic transformation, with chronic gastro-oesophageal reflux disease (GORD) and Barrett's oesophagus (BO) being classified as major risk factors [4, 5]. After prolonged periods of caustic injury to the luminal surface of the oesophagus, 5-13% of patients suffering with GORD will progress onto BO [6]. While the relative risk of developing OAC on a background of GORD and BO is approximately 4.5 and 29.8, respectively [4], the annual risk of developing OAC in a background of BO remains low, at just 0.12% [5].

1.1.1 GORD

Gastro-oesophageal reflux disease (GORD) is a common finding, with population based studies identifying that between 10-20% of adults report at least weekly symptoms in western Europe and the US [7, 8]. However, as there is currently no gold standard test for diagnosis, the presence of GORD has proved extremely difficult to define. The Montreal definition and classification was developed in 2006 to combat confusion in diagnosis and management of the disease. This globally accepted definition states that GORD is *"a condition which develops when the reflux of stomach contents cause troublesome symptoms and/or complications"* [2]. Symptoms of GORD include heartburn (burning sensation in the retrosternal area) and regurgitation. Patients with GORD can fall into 2 distinct categories; Symptomatic GORD (without oesophageal injury) and GORD with oesophageal injury (Figure 1.1).

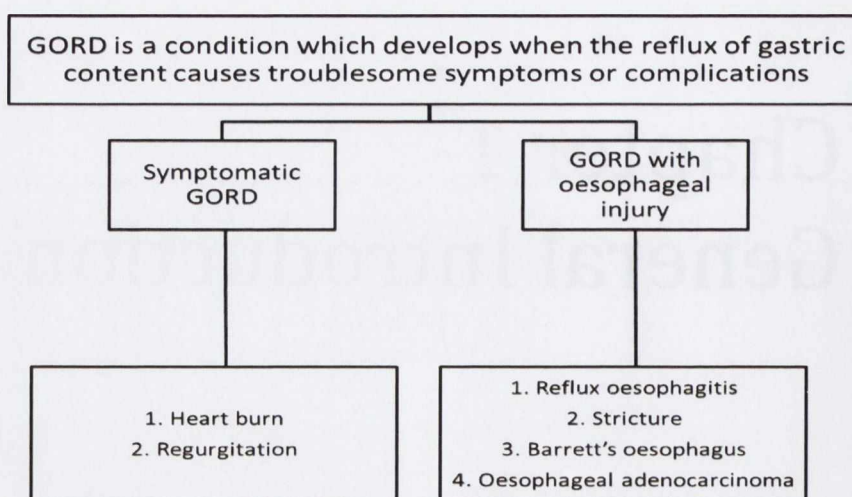


Figure 1.1: Montreal definition and classification system [2].

1.1.1.1 Reflux oesophagitis

Reflux oesophagitis (RO) is the most common complication of GORD [2]. As it can be easily visualised during endoscopy it provides an objective criteria for diagnosis. Histologically, RO is characterised by an increase in immune cell infiltration and basal cell hyperplasia [9]. It is defined by visible breaks in the distal oesophageal mucosa during endoscopy [2]. The Los Angeles classification system provides a method to grade the level of severity of RO. It evaluates the extent of oesophagitis by examining whether mucosal breaks extend between the tops of mucosal folds as shown in (Table 1.1) [10] and has proved to be consistent at predicting outcome of acid reflux therapy. This classification system is used in St. James’s hospital and was therefore used in this study to identify patients with RO. The prevalence of RO has not been widely reported however, a population based study in Sweden stated that reflux symptoms were reported in 40% of the population and RO was diagnosed in 15.5%. Furthermore however, RO was identified in one-third of those who did not report symptoms [11]. Other less common complications of GORD include haemorrhage, stricture, BO and OAC.

Table 1.1: The Los Angeles classification of Oesophagitis [10].

Grade A	One (or more) mucosal break no longer than 5 mm that does not extend between the tops of two mucosal folds
Grade B	One (or more) mucosal break more than 5 mm long that does not extend between the tops of two mucosal folds
Grade C	One (or more) mucosal break that is continuous between the tops of two or more mucosal folds but which involve less than 75% of the circumference
Grade D	One (or more) mucosal break which involves at least 75% of the esophageal circumference

1.1.1.2 Mechanisms of GORD

The underlying mechanism for GORD is not well understood, however factors considered to play a role in development include the transient relaxation of the lower oesophageal sphincter muscle, presence of hiatal hernia, bile reflux and hypotensive lower oesophageal sphincter [12].

Transient lower oesophageal sphincter relaxation (TLOS) is considered a major cause of GORD, accounting for reflux in approximately two thirds of GORD patients [13, 14]. The lower oesophageal sphincter (LOS) is a muscle that surrounds the junction between the oesophagus and the stomach. At rest the LOS remains closed thus preventing gastroesophageal reflux. Upon swallowing the LOS pressure falls to allow the passage of food and liquids, and remains low for approximately 6-8 seconds [15]. TLOS is a different response than swallowing and is considered as a physiological response to gastric distension following a meal, to rid the stomach of excess gas [16]. While studies have not identified an increase in the frequency of TLOS, it has been more frequently associated with acid reflux in GORD patients compared to healthy controls [17].

The LOS is surrounded by the crural diaphragm which contributes to the resting pressure of the LOS [12, 18]. A hiatal hernia is the protrusion of the stomach through the hiatus (opening where the oesophagus protrudes through the crural diaphragm). In some cases the LOS is located in the chest instead of at the level of the diaphragm, thus losing diaphragmatic support during contraction (Figure 1.2). This is known as a sliding hiatal hernia and may be critical to development of GORD [19, 20]. Hiatal hernia lower LOS pressure due to loss of intra-abdominal and diaphragmatic pressure [21].

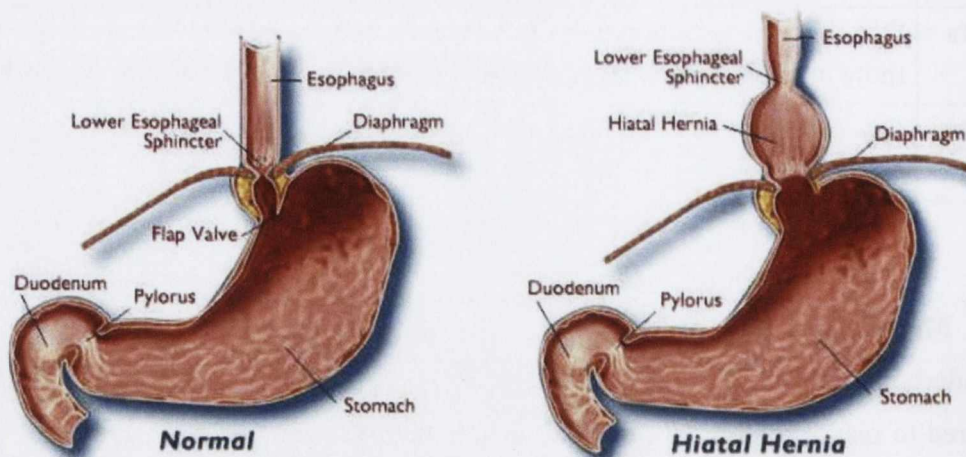


Figure 1.2: Sliding hiatal hernia

1.1.2 Clinical management of GORD

Mild and infrequent reflux symptoms are commonly treated with over the counter medications such as antacids and H₂ receptor antagonists. The aim of these treatment strategies is to reduce gastric acidity. The key advantage of antacids is their rapid onset of action, however, their effect is relatively short lived [12]. As the action of antacids is dependent on neutralisation of gastric acid, their effect is limited by the process of gastric emptying [22]. The time of gastric residence for liquid antacids has been reported to be just 60 min when administered after a meal [22] and thus sustained symptomatic relief may require repeated dosing. The duration of action of H₂ receptor antagonists is longer than traditional antacids; their onset of action however is not as rapid. Thus, they are normally taken before heavy meals and activities likely to induce reflux [12].

Proton pump inhibitors

In patients with moderate to severe symptoms, proton pump inhibitors (PPIs) have become first line treatment. PPIs significantly decrease gastric acid secretion by inhibiting the H⁺K⁺ ATPase pump and thus decrease reflux into the oesophagus. They have been shown to effectively control reflux symptoms and allow oesophageal mucosal healing in oesophagitis [23]. However with advanced grade of RO, there is a limited symptomatic and healing response to PPI treatment [24, 25]. While a complete resolution of oesophageal inflammation can be achieved in these patients, they may still suffer from reflux symptoms [26].

Surgical management

In cases where symptoms cannot be adequately controlled, patients may undergo anti-reflux surgery. The most common form of anti-reflux surgery is laparoscopic nissen fundoplication. This involves wrapping the gastric fundus (upper part) of the stomach around the lower end of the oesophagus, thus re-enforcing the lower oesophageal sphincter. It is a highly effective procedure, with studies reporting that up to 90% of patients achieving a 10 year remission of symptoms [27].

1.1.3 Barrett's Oesophagus

Following chronic exposure to refluxed acid between 5 and 13% of GORD patients will develop Barrett's oesophagus (BO) [6]. It is a premalignant condition that is characterised pathologically by specialised intestinal metaplasia of the distal

oesophagus. The normal stratified squamous epithelium of the oesophagus is replaced by a columnar type cell normally found in the intestine [28]. BO is a common finding in the general population with approximately a 1-2% incidence rate [29]. It is the only known pre-malignant condition of oesophageal adenocarcinoma (OAC). Estimates of the annual incidence rates of OAC in patients with BO have shown considerable variation and range from approximately 0.12%-1% [5, 30-32].

There has been significant controversy around the definition of BO. The current definition from the American Gastroenterological Association (AGA) is *“the condition in which any extent of metaplastic columnar epithelium that predisposes to cancer development replaces the stratified squamous epithelium that normally lines the distal oesophagus”* [33]. While there are three types of columnar epithelium found in BO; gastric-fundic type, cardia type, and intestinal type including goblet cells, only the specialised intestinal type is associated with the risk of OAC [34]. Thus the confirmation of intestinal metaplasia is critical to the diagnosis of BO. However, the British Society of Gastroenterology (BSG), refers to BO as any area of the normal squamous lining which has been replaced by macroscopically visible columnar cells [35]. Thus the presence of intestinal metaplasia is not required for diagnosis. The rationale for this difference is based on the high rate of sampling error during endoscopy, meaning that intestinal metaplasia could be missed upon initial endoscopy.

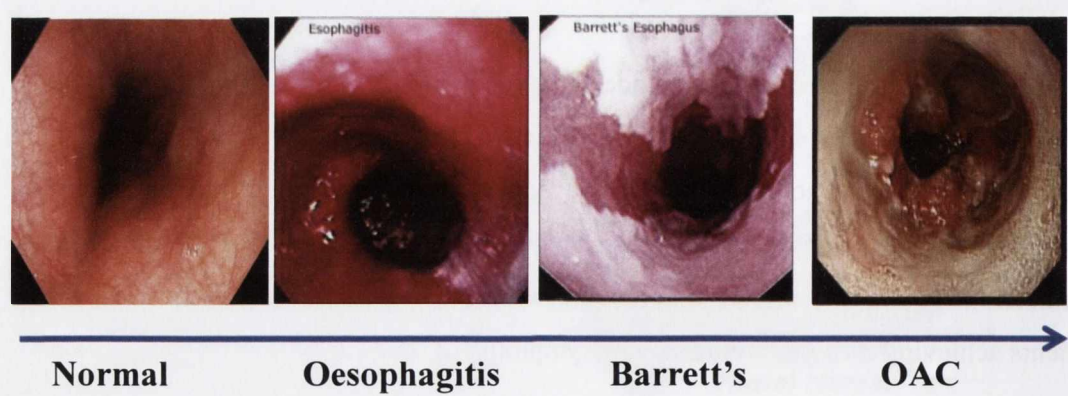


Figure 1.3: Endoscopic images demonstrating Normal, oesophagitis, Barrett's oesophagus and OAC.

Barrett's oesophagus is diagnosed by the combination of the presence of the salmon pink mucosa present at endoscopy (Figure 1.3), and confirmation of specialised intestinal metaplasia on histology. The Prague classification system is a standardised approach to the description of BO based on its circumferential extent (C value) and the maximum extent (M value) above the gastro-oesophageal junction in centimetres (Figure 1.4) [3], and is one of the classification systems commonly used at St James's Hospital. BO can then be further classified into either short- or long-segment disease. Short segment defines intestinal metaplasia less than 3 cm long, whereas long-segment Barrett's is ≥ 3 cm. Patients with long-segment Barrett's have a greater risk of developing OAC, with 65% of OAC arising from long segment BO [36]. In the research setting many studies including this thesis use the presence of villin to identify BO tissue [37]. Villin is an end-differentiated marker of intestinal cells, and needs to be of a sufficient quantity to result in a mature brush border and has been shown to be present in BO tissue [38].

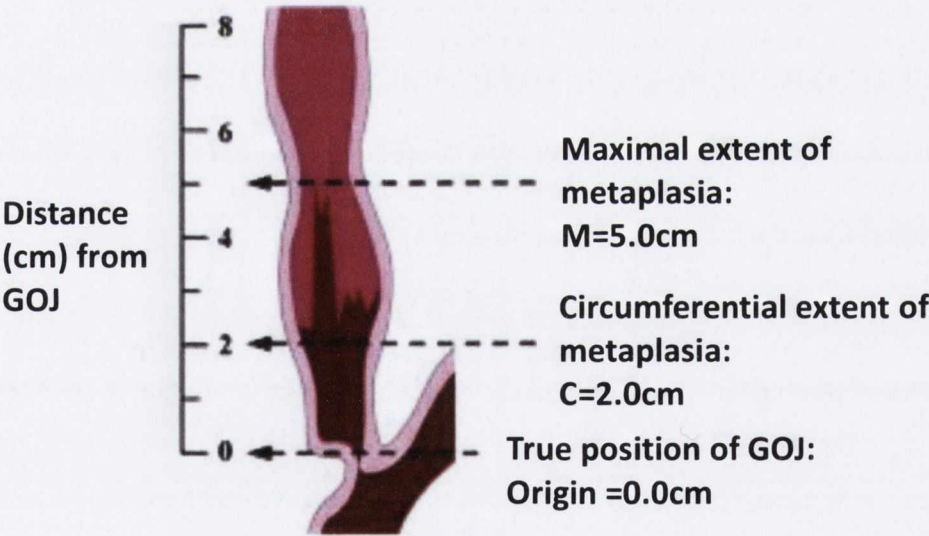


Figure 1.4: Prague Classification system for Barrett's oesophagus.

Example shows area classified as C2M5 (GOJ: gastro-oesophageal junction) [3].

1.1.3.1 Mechanisms of BO

There have been numerous theories about the development of BO in regards to the origin of the columnar epithelium. Initial theories suggested that BO represented the migration of gastric columnar cells from the gastro-oesophageal junction, to replace reflux damaged squamous epithelium [39], however these theories have since been disregarded following studies in animal models [40].

One suggested origin of cells in BO, involves the reprogramming of normal squamous cells into intestinal epithelial cells. During development the oesophagus is lined with a columnar epithelium, through a process of transdifferentiation during late embryogenesis, this is then replaced by a mature squamous epithelium [28, 41]. Treatment of oesophageal squamous cells with acid and bile can lead to the expression of columnar markers [42, 43], thus suggesting a possible reversal in the developmental programming upon acid exposure [28] (Figure 1.5).

Another possibility is that novel stem cells may be recruited to the oesophagus. These stem cells are proposed to originate from the gastric cardia, submucosal glands or even from the circulation (bone marrow derived stem cells) [44]. A study by Quante *et al.*, demonstrated that LGR5⁺ gastric cardia stem cells within BO. They further identified increased Krt19 and TFF2 expression (markers for gastric progenitor cells) in BO. Therefore they suggest that gastric cardia stem cells migrate up from the stomach to form the columnar lined epithelium in BO [44, 45].

However, the most recent and most relevant to this study is the hypothesis that BO develops indirectly as a result of changes in the local tissue microenvironment [13, 22]. Secreted factors, including cytokines such as CXCL8 and IL-1 β , released from stromal cells in response to acid exposure may potentially impact the differentiation of cells within the oesophageal epithelium and may drive the development of BO. It is entirely possible that this occurs in conjunction with the above hypothesis and that the inflammatory milieu present as a result of the immune cell composition directly alters stem cell populations. This inflammatory component will be discussed in greater detail in later sections.

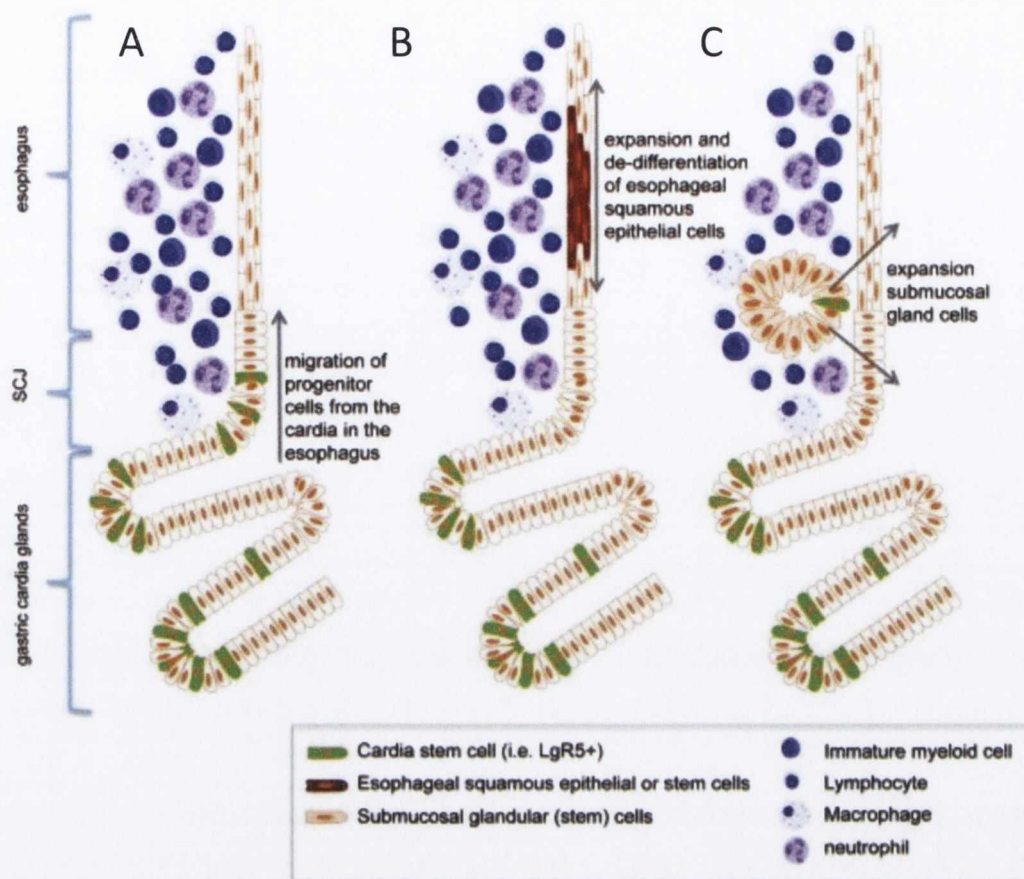


Figure 1.5: Hypothesised cells of origin in BO.

A) Gastric cardia stem cells migrate to the oesophagus. B) Oesophageal squamous epithelial cells de-differentiate and expand to form columnar epithelium. C) Stem cells from submucosal glands expand and give rise to BO. Image adapted from Quante *et al.*, 2012.

1.1.3.2 Clinical management of Barrett's oesophagus

Barrett's management focuses on controlling reflux symptoms and managing cancer development through surveillance programmes. Similar to GORD, BO is controlled with the use of PPIs, with further surgical options (fundoplication) taken for ongoing symptoms.

Surveillance

Guidelines for surveillance programmes suggest endoscopic surveillance every 2 years [46]. If low grade dysplasia is identified surveillance period is shortened. The overall aim of surveillance is for early detection of malignant changes. Studies have demonstrated that cancer detected through surveillance programmes had a lower

staging and were associated with better survival [47]. Ongoing surveillance for BO however, remains controversial as the majority of patients with non-dysplastic Barrett's do not develop oesophageal adenocarcinoma [5]. Epidemiological studies have suggested that up to 95% of oesophageal adenocarcinomas have no prior diagnosis of BO [47, 48]. Stratification of BO patients into high and low cancer risk groups remains a significant clinical challenge.

Proton pump inhibitors

PPIs are used in BO to control reflux symptoms. While isolated reports have suggested regression of BO upon treatment [49], studies have not demonstrated reliable regression or a reduction in the risk of cancer development [28]. Furthermore questions have been raised over the impact on OAC progression. A nationwide study carried out in Denmark revealed an increased relative risk of oesophageal adenocarcinoma or high-grade dysplasia of 2.2 and 3.4 in long-term low- and high-adherence PPI users respectively [50]. The authors suggest that this increased risk could be attributed to the increased mutagenic potential of refluxed bile at alkaline pH [51] [52].

Ablation

If reflux symptoms can be successfully managed, patients frequently undergo ablation techniques to destroy the columnar epithelium. In the presence of a now acid free environment the metaplastic mucosa can be successfully replaced with a histologically normal squamous epithelium [53]. Ablation techniques include argon plasma coagulation, photodynamic therapy, and radio frequency ablation. Studies have demonstrated that ablation can successfully eradicate intestinal metaplasia and significantly reduce the risk of malignancy [54, 55]. For example, a study by Overholt *et al.*, demonstrated that BO patients treated with photodynamic therapy had a significantly lower occurrence of OAC (13%) compared with patients treated with PPIs only (28%) [54]. Similarly clinical trials have demonstrated that BO patients treated with radio frequency ablation have reduced risk of OAC development compared to those treated with PPIs only (4% vs 16.3%) [55].

1.1.4 Oesophageal cancer

Oesophageal cancer is the 8th most common cancer worldwide [56] and is associated with a 5 year survival rate of just 15% [57, 58]. Oesophageal cancer can be divided into two main histological subtypes; oesophageal adenocarcinoma (OAC) and oesophageal

squamous cell carcinoma (SCC) [59]. OAC arises from the specialised intestinal epithelium and occurs at the junction of the oesophagus and the stomach. SCC arises from the squamous epithelium and occurs in the upper two thirds of the oesophagus [60]. SCC represents the majority of oesophageal cancer cases worldwide [60, 61]. In western countries however, OAC is rapidly increasing in incidence and is fast becoming the leading form of oesophageal cancer in the west [62]. In Ireland OAC has risen by 27% in the last 15 years and has been accompanied by a decrease in the rates of SCC which has dropped 4% in the last 15 years (national cancer registry Ireland).

1.1.4.1 Risk factors

As mentioned, OAC typically develops from the specialised intestinal metaplasia of Barrett's oesophagus [46, 63]. Thus GORD and BO are critical risk factors for its development. Barrett's oesophagus remains the leading risk factor for OAC development carrying a 30 fold increase risk of developing OAC [4]. Further risk factors for OAC development include; obesity, Caucasian ethnicity, male gender and increased age [64, 65].

In an Irish cohort obesity has been associated with a 4-fold risk of OAC compared to normal weight controls. The study by Ryan *et al.*, showed that males with a BMI in the top quartile had a nine fold increase risk of developing OAC compared to normal weight counterparts, whereas the risk for overweight females was 3.6 fold higher than normal weight counterparts [66]. Conversely, meta-analysis have shown significant inverse association between *H. pylori* infection (OR: 0.57) and OAC [67, 68]. Similar associations were identified with *H. pylori* infection and BO development (OR: 0.46) which may in part contribute to reduced OAC development [69].

1.2 Inflammatory mechanisms behind the GORD-BO-OAC sequence

The cellular pathogenesis of injury, inflammation, metaplasia, dysplasia and adenocarcinoma is unclear. In terms of GORD, it was initially assumed that cellular damage was a direct result of caustic injury at the luminal surface of the oesophagus due to chronic acid and bile reflux [70, 71]. However, more recent evidence suggests that the inflammation itself may be a key trigger. Souza *et al.*, alluded to the importance of the inflammatory microenvironment by demonstrating that refluxed acid did not directly damage the epithelial layer of the oesophagus, but instead induced the

production of chemokines and cytokines including CXCL8 and IL-1 β , which significantly influenced and altered T cell migration to oesophageal tissue [72]. Thus suggesting that T cells may initiate the inflammatory response and damage in GORD, making them pivotal players in oesophageal disease.

1.2.1 Antigen recognition by T cells

T cells are key players in the adaptive immune response and can be distinguished from other immune cells by the presences of the T cell receptor complex. The T cell receptor (TCR) is a membrane bound glycoprotein composed of two disulphide linked chains, α and β [1, 73]. Each chain folds into two Ig-like domains (a constant region and a variable region) which are structurally similar to the Fab portion of an immunoglobulin molecule (Figure 1.6). The variable region contains hypervariable regions which confer antigen specificity. However, these chains have short cytoplasmic tails and with no intrinsic signalling activity. The association of the TCR with the CD3 complex provides a mechanism for intracellular signal transduction. The CD3 complex is composed of four transmembrane chains CD3 γ , δ , ϵ , and ζ . The α chain of the TCR is thought to interact with one CD3 $\delta\epsilon$ dimer and one ζ dimer, while the β chain of the TCR is thought to interact with one CD3 $\gamma\epsilon$ dimer. Each CD3 chain has one or more immunoreceptor tyrosine based activation motif (ITAM), which is vital for signal transduction following receptor activation. In addition to the TCR and CD3 complex, the majority of T cells also express the membrane bound proteins CD4 or CD8. These molecules act as co-receptors for the MHC molecule and play a role in initiating intracellular signalling. The cytoplasmic tails of the CD4 and CD8 molecules are associated with the protein tyrosine kinase Lck. Phosphorylation of the ITAMs on the CD3 chains, allows for the recruitment and activation of ZAP70 to the TCR signalling complex. ZAP70 is another protein tyrosine kinase, which in turn propagates the signal for T cell activation. The TCR cannot directly recognise free antigen. The majority of T cells recognise only peptides displayed in the peptide binding groove of an MHC molecule. Thus the antigen must be taken up, processed and presented to the T cell [1]. There are two classes of MHC molecule and in general, CD4⁺ T cells recognise antigen presented by MHC class II, whereas CD8⁺ T cells recognise MHC class I.

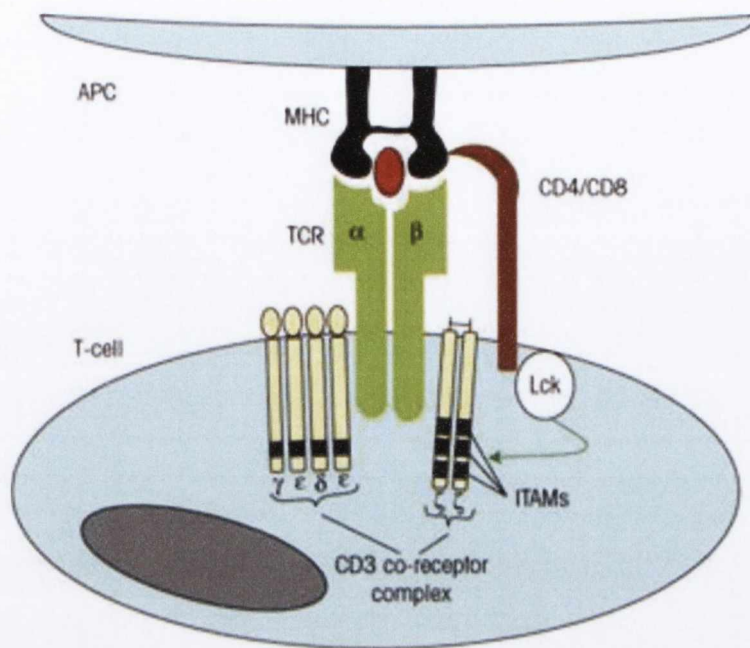


Figure 1.6: T cell receptor complex and signalling [1].

1.2.2 T cell development

Developing T cells within the thymus start off as “double negative” thymocytes, and do not express CD3, CD4 or CD8 on their cell surface [1, 74]. These cells then give rise to the two lineages; the $\alpha\beta$ T cells and the $\gamma\delta$ T cells. At this point in development the $\alpha\beta$ T cells develops into “double positive” thymocytes expressing CD3 and both CD4 and CD8, while the $\gamma\delta$ T cells express CD3 but neither CD4 or CD8 and are exported to the circulation. The $\alpha\beta$ T cells then divide in the thymus where a small percentage can interact with self-peptide:self-MHC complexes (positive selection) and lose expression of either CD4 or CD8. This then creates two “single positive” populations which are exported to the circulation as mature $CD4^+$ or $CD8^+$ T cells [1, 74].

Naïve T cells

Once naïve T cells enter the circulation they continuously recirculate between the blood and the secondary lymphoid organs. A number of markers can be used to identify naïve T cells; these include CD45RA, CD62L and CCR7 [75]. CD45 is a tyrosine phosphatase that is found on the surface on lymphocytes and can differentiate between naïve and memory cells. Naïve T cells express the CD45RA isoform, which is

downregulated upon activation and replaced by CD45RO. CD62L, also known as L-selectin and CCR7, a chemokine receptor are two molecules that play a role in the recirculation of T cells between the blood and lymphoid organs [76, 77]. Naïve T cells can therefore be described as $CD45RA^+CD62L^+CCR7^+CD45RO^-$.

Effector and memory T cells

Once a T cell encounters its cognate antigen, presented on the surface of a mature dendritic cell it undergoes clonal expansion and differentiation. It gives rise to effector T cells of the same antigen specificity, which then migrate to the site of infection [73]. $CD8^+$ T cells recognise antigen presented by MHC class I and differentiate into cytotoxic effector T cells. $CD4^+$ T cells recognise antigen presented by MHC class II and differentiate into distinct effector subsets. These include Th1, Th2, Th17, Tfh, Th9 and Th22 cells. Again a number of markers can be used to identify activated and memory cells; these include CD69, CD45RO and CD62L. CD69 is expressed on T cells shortly after activation acting as a co-stimulatory molecule leading to proliferation, cytokine secretion or cytotoxicity [78]. While at sites of chronic inflammation however, CD69 can be constitutively expressed, under normal conditions its expression is transient and it is not seen on memory T cells [79]. CD45RO as mentioned is upregulated upon activation and is found on both effector and memory T cells [75]. Furthermore, memory T cells express CD62L which allows for their continuous recirculation through the blood and lymph nodes. Memory cells can be further divided based on CCR7 expression into central memory ($CD62L^+CCR7^+CD45RO^+CD45RA^-$) and effector memory ($CD62L^-CCR7^+CD45RO^+CD45RA^-$).

1.2.2.1 CD4 effector subtypes

Upon activation, CD4 cells clonally expand and can differentiate into distinct sub-populations including Th1, Th2, and Th17. Each sub-population is associated with unique cytokine expression and their development is driven by distinct signals and transcription factors.

Th1/Th2

IFN- γ is the prototypical Th1 cytokine, however they also secrete IL-2, TNF- α and GM-CSF and lymphotoxin [80] and are thought to promote a pro-inflammatory and cell mediated immunity. The presence of IFN- γ and IL-12 during T cell activation

promotes Th1 development via the activation of STAT1 and STAT4 respectively. STAT1 induces the expression of the transcription factor t-bet, thus promoting the production of Th1 cytokines [73]. Upon antigen recognition, these cells will now produce copious amounts of IFN- γ , thus further promoting Th1 differentiation. Th2 cells produce the cytokines IL-4, IL-5, IL6, IL-10 and IL-13 and are associated with a more humoral based immune response [80]. IL-4 provides key signals for Th2 development through the activation of STAT6. STAT6 promotes the transcription factor GATA3, which induces the expression of the characteristic Th2 cytokines [81]. The developmental routes for Th1 and Th2 cells appear to be mutually antagonistic. For example, IL-4 which is critical for the development of the Th2 lineage actively inhibits IFN- γ release and Th1 development, likely through binding to the IL-4 receptor found on mature Th1 cells. Conversely production of IFN- γ inhibits the proliferation of Th2 cells [82]. Experimental models have suggested that a shift from a Th1 to Th2 response may play an important role in cancer development and progression. In a murine model of B cell lymphoma, Lee *et al.*, demonstrated that mice susceptible to tumour development displayed a predominantly Th2 response; whereas mice resistant to tumour challenge developed a Th1 response [83]. Furthermore, a mouse model of renal cell carcinoma demonstrated a combined loss of Th1 populations and increase in Th2 with tumour progression [84]. Similarly in several human cancers Th2 cells have pro-tumourigenic effects and are related to poor prognosis and reduced survival [85]. In pancreatic cancer for example, Th2 cells accelerate disease progression and reduce patient survival [86]

Th17

Th17 are characterised by the production of IL-17A, IL-17F, IL-21 and IL-22. They develop in the presence of IL-6 and TGF- β and absence of IL-4 and IL-12 [73]. During Th17 development, IL-21 is initially produced and acts in an autocrine fashion to activate STAT3, which activates the transcription factor ROR γ T, driving Th17 development. Th17 cells have been associated with the induction of many autoimmune disorders including multiple sclerosis, inflammatory bowel disease and rheumatoid arthritis [87, 88]. One of the main physiological functions of Th17 cells is to promote barrier immunity at mucosal surfaces such as the gut and skin [89]. Studies suggest that Th17 cells are increased within the tumour microenvironment, and are involved in tumour progression [90-92]. While some studies support an anti-tumour role for Th17 cells, showing they can mediate tumour rejection [93, 94]; others suggest a tumour

promoting effect showing Th17 cells can promote angiogenesis and correlate with blood vessel formation in a number of cancers [95]. Th17 cells are increased in oesophageal cancer patients compared to healthy controls in both blood and tissue [96, 97], and have been associated with disease stage.

Tregs

Tregs cells can broadly be divided into “natural” Tregs (nTreg) or inducible Tregs (iTreg). Natural Tregs develop in the thymus and express high levels of CD25, and the transcription factor FoxP3 [98]. Inducible Tregs differentiate outside the thymus into CD25⁺ and FoxP3⁺ cells following antigen stimulation in the presence of immunoregulatory cytokines such as TGF- β , IL-10 and IL-4 [99]. Regulatory T cells (Tregs) play an important role in immune homeostasis, suppressing the activation, proliferation and effector functions of a variety of immune cells [100]. The mechanisms by which Tregs suppress immune cell function have not yet been fully characterised; however, it is known that Tregs can induce an immunosuppressive environment through the secretion of IL-10 and TGF- β [101]. Furthermore, it has been demonstrated that Tregs can reduce antigen presenting capacity of APCs [101]. High numbers of Tregs have been reported in a variety of malignancies including gastric and oesophageal and are associated with poor survival [102-104]. [102-104][102-104][102-104][102-104][102-104][102-104][102-104][102-104][102-104][102-104] Thus it is suggested that increased levels of Tregs can suppress an efficient anti-tumour immune response [102-104].

1.2.3 Inflammatory profile in GORD, BO and OAC

Studies have demonstrated increased levels of IL-1 β , IFN- γ and CXCL8 mRNA in oesophagitis tissue compared to controls [105-107]. IL-1 β is produced by oesophageal epithelial cells upon exposure to acid reflux [107-110] and is increased in the affected oesophageal mucosa of GORD patients. It has been recognised as an important driving factor in the progression from GORD to BO and OAC; recently Quante *et al.*, overexpressed human IL-1 β in the oesophagus to create a murine model for the oesophagitis-metaplasia-adenocarcinoma sequence [45]. Furthermore, animal models of chronic oesophagitis, have demonstrated significantly increased levels of pro-inflammatory mediators TNF- α and CCL3 [111, 112]. These findings have been suggested to demonstrate a predominantly Th1 like cytokine response in oesophagitis tissue.

Studies have demonstrated that BO tissue displayed increased levels of IL-10 and IL-4 exclusively [107]. IL-4 has been suggested as a potential modulator of BO, potentially driving the metaplastic transformation [113]. In a primary cell model, Shan *et al.* demonstrated that IL-4 leads to the down regulation of squamous cell markers, involucrin and keratin 13, while up-regulating columnar markers, keratin 7 and keratin 8 [113]. Furthermore, immunohistochemical studies by Moons *et al.*, demonstrated higher levels of Th2 effector cells (plasma and mast cells) in BO tissue compared to oesophagitis tissue suggesting BO is characterised by a pronounced humoral and Th2 immune response.

A further inflammatory shift to a more Th1 response is suggested to occur in OAC with levels of pro-inflammatory cytokines such as IFN- γ , IL-2, IL-1 β and the chemokine CXCL8 increased compared to healthy controls [105, 106]. Th1 associated cytokines, like IFN- γ , are known to stimulate a potent anti-tumour immune response. IFN- γ enhances tumour cell immunogenicity through the upregulation of MHC class I genes [114]. Furthermore, recombinant IFN- γ has been shown to have anti-proliferative [115, 116], anti-angiogenic [117, 118], and pro-apoptotic effects on cancer cells [119]. Another important aspect of Th1 cells is their ability to activate cytotoxic T lymphocytes (CTLs). CD8⁺ CTLs are capable of directly killing tumour cells and indeed higher numbers of tumour infiltrating CD8⁺ T cell has been correlated with improved patient survival in numerous cancers including oesophageal, colorectal and ovarian [120-122]. Th2 like cytokines, on the other hand are considered to be pro-tumourigenic. IL-10 for example, can not only inhibit anti-tumour immune response through the inhibition of Th1 responses, it is also known to promote angiogenesis [123], thus promoting tumour progression. Thus the switch to a Th2 profile in BO may predispose to tumour development and it should be of vital importance to identify the mechanisms behind these phenotypic switches. While these previous studies have examined cytokine profile in oesophageal tissue, the distinct T cell populations have not yet been examined. As such the overall aim of this thesis was to characterise T cell profile in the progression from GORD-BO-OAC and identify potential immunotherapeutic targets to slow or prevent disease progression.

1.2.4 T cell immunotherapies

Immunotherapeutic approaches to cancer treatment have been around for over a century and aim to harness both the natural specificity of the adaptive immune response as well

as the diverse and cytotoxic response of the innate and adaptive immune response [124]. Several approaches to T cell based immunotherapies have been identified to date and have shown promise in clinical trials. They include; checkpoint inhibitor and immune modulators, adoptive T cell transfer, tumour infiltrating lymphocyte (TIL) therapies and T cell receptor based therapies.

Checkpoint inhibitors

The use of immune checkpoint inhibitors aims to overcome the inhibitory effects of the tumour microenvironment on T cells and have shown promising effects in clinical trials. CTLA-4 is a co-inhibitory molecule expressed following T cell activation. It binds to members of the B7 family expressed on APCs to inhibit further T cell activation and proliferation thus attenuating the immune response [125]. As such *ctla4*^{-/-} mice display an aggressive and lethal lymphoproliferative disorder [126]. Ipilimumab is a humanised mAb that binds CTLA-4 and inhibits signalling thus significantly increasing the number of activated T cells. It has been shown to significantly enhance anti-tumour immune responses. In phase III clinical trials Ipilimumab demonstrated clinical efficacy in advanced melanoma and has been approved by the FDA for the treatment of metastatic melanoma [127]. Since then, Ipilimumab and other CTLA-4 inhibitors (tremelimumab) have demonstrated promising results in clinical trials in several cancer types including colorectal, renal cell carcinoma, prostate cancer, non-small cell lung carcinoma and gastric/gastro-oesophageal junction cancer [128]

PD-1 (programmed cell death receptor -1) is another inhibitory receptor involved in establishing and maintaining immune tolerance. Similar to CTLA-4, PD-1 is upregulated following T cell activation and upon binding to its ligands PD-L1 and PD-L2 (also members of the B7 family) inhibits further T cell function and proliferation [129]. PD-L1 has been found to be expressed on a variety of tumour types, these include non-small cell lung [130], melanoma [131], gastric [132] and hepatocellular carcinoma [133]. Furthermore, studies have identified gastric cancer, renal cell carcinoma and hepatocellular carcinoma that higher levels of PD-1 are associated with poor prognosis [134-136]. Both anti PD-1 and anti PDL-1 mAbs have entered clinical trials for the treatment of advanced solid tumours. A phase I study for the anti PD-1 mAb (MDX-1106) in solid tumours, demonstrated anti-tumour activity, with a complete response observed in one colorectal patient [137]. The anti PD-L1 mAb has also undergone phase I clinical trials for the treatment of advanced solid tumours. Results

demonstrated that blockade of PD-L1 induced tumour regression and stabilisation in patients with non-small cell lung cancer, melanoma and renal cell cancer [138]. Furthermore the first anti PD-1 therapy, KEYTRUDA (pembrolizumab), was recently approved by the FDA for the treatment of advanced melanoma and has demonstrated prolonged progression free survival compared to patients treated with ipilimumab [139] and was furthermore associated with less toxicity than.

TIL therapies

Adoptive cell transfer (ACT) of tumour infiltrating lymphocytes (TIL) has shown significant clinical benefit in metastatic melanoma. Clinical responses were observed in 50-70% [140], and complete tumour regression was demonstrated in 22% [141], of patients with metastatic melanoma. One of the disadvantages of adoptive transfer of TILs is the difficulty in isolating and expanding sufficient numbers of TILs for therapy [142]. Furthermore, adoptive transfer of TIL is MHC restricted. Since the majority of tumours can alter their MHC expression to evade immune response, TIL transfer has limited response being largely restricted to melanoma [143, 144]. Alternative methods of ACT are currently being explored and overcome these obstacles. T cells can be engineered to specifically recognise and target tumour associated antigens through the expression of transgenic T cell receptors or chimeric antigen receptors (CAR).

Transgenic T cell receptor

Similar to TIL transfer, the generation of transgenic T cell receptors (tgTCR) involves the isolation of tumour specific T cells from a patient and their *ex vivo* expansion. This allows for the isolation of genes encoding the TAA (tumour associated antigen) specific TCR and their subsequent transduction into T cells isolated from patient blood [145]. These newly formed tumour specific T cells can then be expanded in vitro and re-infused into the patient. Thus this method overcomes the difficulty in obtaining sufficient numbers of TILs. Adoptive transfer of T cells expressing a TCR specific for the NY-ESO-1 antigen has been demonstrated to result in clinical responses in synovial cell sarcoma and melanoma patients [145, 146]. A study by Morgan *et al.*, demonstrated sustained levels of T cells expressing the tgTCR for between 2 months and 1 year post infusion, and demonstrated regression of metastatic lesions in melanoma patients [145]. Once again however, this method is MHC restricted and the affinity of the TCR to the MHC complex will affect the efficacy of treatment.

Chimeric antigen receptors

The production of chimeric antigen receptors (CAR) has overcome the difficulties with regards to MHC restriction. CARs are composed of an extracellular antigen recognition domain, and an intracellular signalling domain that mediates T cell activation [147]. The binding affinity of CAR T cells is similar to that of antibodies, so not only overcomes MHC restriction, but also increases binding affinity [148].

1.2.5 Potential T cell based therapies in GORD-BO-OAC

To date T cell based immunotherapies for GORD, BO and OAC do not exist. As such the overall aim of this thesis was to identify potential immunotherapeutic targets to alter T cell function and profile in this progression. This can be broken down into two main sections. Firstly we hypothesise that altered chemokine profiles in the oesophageal microenvironment may lead to the differential recruitment of distinct T cell subsets, thus promoting disease progression. As such we aim to identify potential chemokine pathways responsible for T cell recruitment, thus allowing for therapeutic intervention. Secondly we hypothesise that factors within the oesophageal microenvironment may alter T cell metabolism thus affecting T cell activation and profile. We aim to characterise metabolic profile of oesophageal infiltrating T cells and ascertain whether changes to the local microenvironment can drive characteristic T cell profiles observed in the GORD-BO-OAC sequence through alterations in T cell metabolism.

1.3 Chemokines

Chemokines are a large family of proteins that promote the directional migration of cells through a concentration gradient. They signal through the activation of G-protein coupled receptors (GPCRs) and promote the accumulation of cells at the source of chemokine production [149, 150]. While the most well-known role of chemokines is in host defence and immunity, they also play vital roles in embryonic development, wound healing, angiogenesis and growth and metastasis of tumours [150].

1.3.1 Chemokine structure

Chemokines are defined by the presence of four conserved cysteine residues and are classified into groups depending on the positioning of the first two residues; C, CC, CXC or CX₃C chemokines. If the first two cysteine residues are adjacent to each other, chemokines are classified as CC. Chemokines that have an intervening amino acid

between the first two cysteines are classified as CXC chemokines. While CX3C chemokines are defined by three intervening amino acids between the first two cysteines [149]. While chemokines can have varying degrees of sequence homology, studies have revealed their 3 dimensional structures to be remarkably conserved. They all contain the same monomeric fold which consists of; a disordered N-terminal domain known to contain key signalling domains [151, 152], three β strands, and a carboxy (C) terminal domain [153]. The fold and core structure of chemokines is largely stabilised by disulfide bonds between the four cysteine residues. The N-terminal domain has emerged as playing a vital role on receptor binding and activation. Modification of the N-terminus has been reported to create chemokines without signalling capabilities. For example, modification of the N-terminal of CCL2 can produce a high affinity antagonist to the receptor CCR2 [154, 155]. Other regions are also thought to participate in receptor binding; the β strands in CCL2 and CXCL10 are known to directly bind the chemokine receptor and contribute to high affinity binding [151, 156]. It is speculated that the core domain (everything bar the N-terminus and C-terminus) of chemokines bind to the extracellular loops of the chemokine receptor, correctly positioning the N-terminus signalling domain of the ligand [157].

1.3.2 Chemokine receptors and signalling

As mentioned, chemokines signal by binding to the seven transmembrane G- protein coupled receptors [157]. Chemokine receptors are classified based on the subfamily of chemokines to which they bind i.e. CCR, CXCR [149]. To date up to 50 chemokine ligands and 19 functional receptors have been identified. There is significant redundancy in the chemokines system; chemokine receptors rarely bind to just a single ligand, and multiple chemokines bind to more than just one receptor (Table 1.2) [158]. Interestingly, receptors only bind chemokines from within the same subfamily for example; CXCR2 can bind to seven different chemokine ligands however, they all belong to the CXC chemokine family. Competitive binding across subfamilies is rare [1, 149]. Like all GPCRs, chemokine receptors contain seven transmembrane spanning regions connected by 3 extracellular loops. Within the receptor, structure function studies have identified that the N-terminal domain and one or more extracellular loop of the receptor are involved in ligand binding [159]. Truncation of the N-terminal region of CCR5 can decrease affinity for its ligands CCL5 and CCL4 [160, 161]. Chemokine recognition and receptor activation are thought to occur in a two-step model [149, 162]; firstly the core domain of the chemokine specifically recognises and

binds the receptor N-domain, allowing the ligand to undergo conformational change. This then allows the N-terminal signalling domain of the ligand to interact with the extracellular loops of the receptor allowing for full receptor activation [159]. Activation of the receptor induces the exchange of GDP for GTP in the α -subunit of the G proteins. The G proteins now dissociate from the receptor and initiate downstream signalling [163].

1.3.3 Chemokine receptor expression on T cells

T cells are an extremely migratory cell type and during the course of an immune response must acquire the capability to migrate to different sites allowing for an appropriate immune response. For example naïve T cells continuously recirculate through the blood and secondary lymphoid organs in search of their cognate antigen. Upon antigenic stimulation, naïve cells proliferate and differentiate into effector and memory T cells which must acquire new migratory capabilities. While effector T cells must be able to enter any non-lymphoid tissue where the pathogen exists [76], memory T cells must be able to reach non-lymphoid tissue to protect against a secondary challenge, but they must also be able to enter lymphoid organs for clonal expansion [164]. The expression of chemokine receptors on T cells has been demonstrated to regulate T cell trafficking, and the differential chemokine receptor expression on T cells has been shown to be capable of distinguishing distinct T cell subsets.

Naïve T cells

As mentioned, naïve T cells continuously recirculate between the lymph node and the blood. This occurs via the high endothelial venules (HEVs) found exclusively in secondary lymphoid organs such as the lymph node [76, 77]. Naïve T cells express the receptors CD62L and CCR7 which bind to the ligands PNAd (peripheral lymph node addressin) and CCL21 found on HEVs. CD62L is responsible for the initial adherence of T cells to HEVs [165], whereas CCR7 mediates firm adhesion and diapedesis [75, 166]. Naïve T cells also express CXCR4, however its function in the immune response is not yet clear [167].

Effector T cells

A study by Langenkamp *et al.*, demonstrated that following T cell activation, naïve T cells downregulated CCR7 and CXCR4. Loss of CCR7 was shown to correlate with T cell effector function [168]. Immediately following activation CXCR3 was upregulated

on all cells, thus acting as an early activation marker. Furthermore following activation, T cells polarise towards distinct subsets, with characteristic chemokine receptor expression.

Memory T cells

Expression of CCR7 has been shown to distinguish between the two memory T cell populations [169]. CCR7⁺ memory cells express lymph node homing markers, such as CD62L and lack immediate effector function. These central memory T cells have a similar migratory pathway to naïve T cells and can enter secondary lymphoid organs where they can stimulate dendritic cells upon secondary stimulation. CCR7⁻ memory cells (effector memory) on the other hand, migrate towards inflamed tissue and display immediate effector functions [169]. Migration of effector memory cells occurs via the expression of tissue specific chemokine receptors [76].

Table 1.2: Chemokine receptors and their ligands. Adapted from Allen *et al.*, 2007

Receptor	Ligands
CCR1	CCL3,CCL5,CCL7,CCL13,CCL14,CCL15,CCL16,CCL23
CCR2	CCL2,CCL7,CCL8,CCL13,CCL16
CCR3	CCL5,CCL7,CCL8,CCL11,CCL13,CCL15,CCL16,CCL24,CCL26,CCL28
CCR4	CCL17,CCL22
CCR5	CCL3,CCL4,CCL5,CCL8,CCL11,CCL14,CCL16
CCR6	CCL20
CCR7	CCL19,CCL21
CCR8	CCL1
CCR9	CCL25
CCR10	CCL27,CCL28
CXCR1	CXCL6,CXCL7,CXCL8
CXCR2	CXCL1,CXCL2,CXCL3,CXCL5,CXCL6,CXCL7,CXCL8
CXCR3-A	CXCL9,CXCL10,CXCL11
CXCR3-B	CXCL4,CXCL9,CXCL10,CXCL11
CXCR4	CXCL12
CXCR5	CXCL13
CXCR6	CXCL16
CXCR7	CXCL12
CX ₃ CR1	CX ₃ CL1

Th1/Th2 subsets

Differential chemokine receptor expression can differentiate between T helper subsets. CXCR3 and CCR5 are preferentially expressed on Th1 cells, whereas Th2 cells preferentially express CCR4 and CCR3 allowing for selective migration in response to the corresponding chemokines [164, 170]. CCR5 is a receptor for CCL5, CCL3 and CCL4 and is preferentially expressed on recently activated and Th1 cells [171]. CXCR3 binds to the chemokines CXCL9, CXCL10 and CXCL11 to again induce the migration of activated T cells [172-174].

CCR3 is the chemokine receptor for CCL11, CCL24, CCL5, CCL8, CCL7, and CCL13 and are selectively expressed on Th2 cells producing IL-4. Th2 cells also express the receptor CCR4, which responds to the chemokine CCL17. However, CCR4 has also been shown to be present on cells that do not produce IL-4 and may not therefore be a faithful Th2 marker [170].

Th17

CCR6 is the only known receptor for CCL20 and is expressed on Th17 cells [171, 175]. However, other studies have reported that Th17 cells may express a far more diverse range of chemokine receptors, showing expression of CCR6 as well as CCR5, CCR4 and CCR2 [176].

1.3.4 Chemokines in the GORD-BO-OAC sequence

A novel mechanism has been described whereby exposure of epithelial cells to refluxed acid can lead to oesophageal damage as seen in GORD. Studies have reported upregulation of CXCL8 expression, at both the mRNA and protein level following exposure to refluxed bile acids. This has been demonstrated to induce the migration of inflammatory cells to oesophageal tissue [72, 177, 178]. Thus, it is suggested that chemokines mediate inflammation and cellular damage through the recruitment of immune cells to oesophageal tissue. Elevated levels of CXCL8, CCL2 and CCL5 have been reported in oesophagitis tissue compared to healthy tissue and have been shown to correlate with level of severity [179]. However despite their importance in the initiation of inflammation in oesophagitis, the chemokine profile in the oesophagitis-metaplasia-carcinoma sequence has not been studied in detail to date. Studies have identified increased levels of CXCL8 in Barrett's and OAC tissue [180, 181]. Furthermore, it has been demonstrated that treatments for reflux, including anti-reflux surgery and the PPI

omeprazole can decrease CXCL8 expression in oesophagitis and BO [178, 180]. In oesophageal squamous cell carcinoma, CCL17 and CCL22 have been reported to induce the migration of regulatory T cells via the activation of CCR4 and thus impair anti-tumour immunity [182]. Similar findings have been observed in gastric cancer [183], however to date, it is not known if the same holds true for OAC.

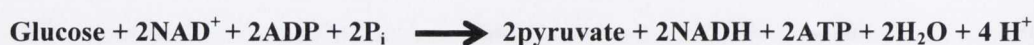
While BO is associated with a Th2 cytokine profile, which is thought to promote the metaplasia-carcinoma sequence however, the mechanism for accumulation of Th2 cells within BO tissue has not yet been described. It is possible that the chemokine profile in the local Barrett's microenvironment may contribute to the distinct T cell polarisation observed via recruitment of Th2 cells, rather than the expansion of already resident T cells. A similar mechanism is seen to occur in Hodgkin's lymphoma; the malignant Reed Sternberg cells express a range chemokines such as CCL17 and CCL22 causing the local accumulation of Th2 cells [184]. Similarly in asthma, a Th2 mediated disorder, T cells infiltrating bronchial biopsies have been shown to be IL-4⁺ and express CCR4. Furthermore, the ligands CCL17 and CCL22 were found to be expressed by airway epithelial cells following antigen challenge [185], thus providing a mechanism for recruitment of Th2 cells to the lung. One of the aims of this thesis was therefore to characterise the chemokine profile in the local oesophageal microenvironment, and to identify chemokine pathways that may contribute to the distinct T cell phenotype observed at each disease stage.

1.4 Metabolism

Multiple factors, including nutrients in the local oesophageal microenvironment have the ability to alter T cell metabolism, phenotype and function. Previous work from our department identified an enhanced metabolic profile in BO and OAC tissue [37]. Thus these cells may use substantial amounts of nutrients, creating a nutrient poor environment for infiltrating T cells. This may impact T cell phenotype and function through altered metabolism, and may therefore play a role in the distinct T cell profiles observed in GORD, BO and OAC.

1.4.1 Anaerobic glycolysis

Under conditions of limited oxygen availability, cells generate ATP through the conversion of glucose to lactate through the process of anaerobic glycolysis. The glycolytic pathway was first elucidated in 1940, and is the metabolic reaction whereby one molecule of glucose is converted to two molecules of pyruvate. This reaction occurs in the cytoplasm of the cell and results in the net production of 2 adenosine triphosphates (ATP) [186, 187]. The overall reaction is as follows:



Glycolysis occurs in a complex set of chemically coupled phosphorylation reactions as described in Figure 1.7. In the initial stages of glycolysis, glucose enters the cell and is phosphorylated by hexokinase to glucose-6-phosphate (G6P), isomerized by phosphoglucose isomerase (PGI) to fructose-6-phosphate (F6P), phosphorylated by phosphofructokinase (PFK) to yield fructose-1,6-biphosphate (FBP), which is then cleaved by aldolase to form two molecules; glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP), which can be interconverted by an isomerase reaction. These initial reactions use 2 molecules of ATP and can be thought of as the energy investment phase. During the later “pay off” phase of glycolysis, GAP undergoes oxidation by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to form the high energy 1,3-bisphosphoglycerate (1,3-BPG), dephosphorylated by phosphoglycerate kinase (PGK) to produce 3-phospho-glycerate (3PG) and one molecule of ATP. Following isomerisation by phosphoglycerate mutase (PGM) to 2-phosphoglycerate (2PG), dehydration by enolase to the high energy molecule phosphoenolpyruvate (PEP), a final dephosphorylation step by pyruvate kinase yields one molecule of pyruvate and a second molecule of ATP [186]. Overall these later stage produce 4 molecules of ATP per molecule of glucose, thus generating a net production of 2 ATP per glucose molecule [186, 187]. The pyruvate generated through glycolysis can then be further processed by 2 possible mechanisms. Under aerobic conditions, it can be completely oxidised via the citric acid cycle or alternatively under anaerobic conditions pyruvate will be reduced to lactate by the enzyme lactate dehydrogenase (LDH) oxidising the NADH molecule to NAD^+ to further fuel glycolysis. This process is termed “anaerobic glycolysis”.

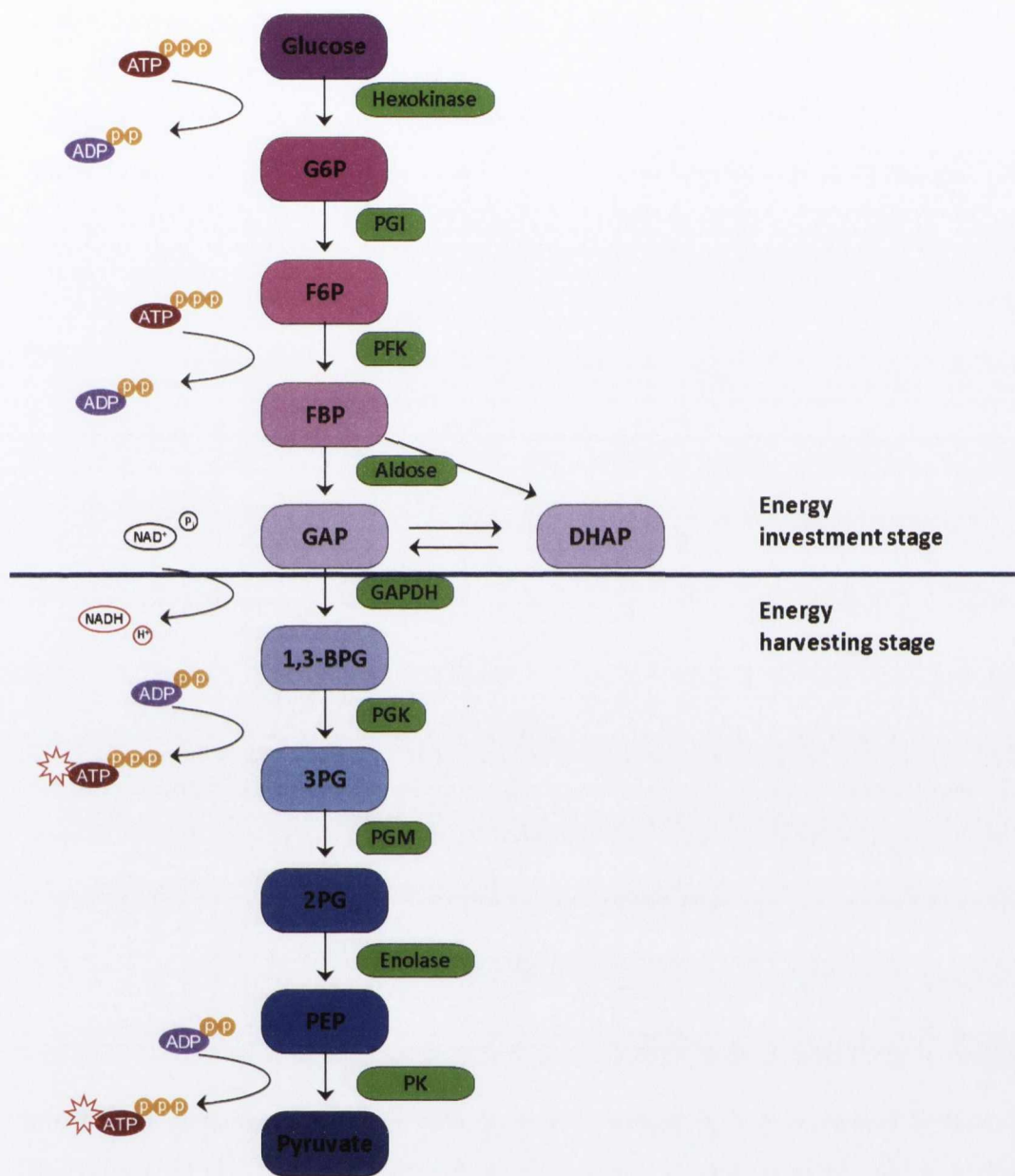


Figure 1.7: Schematic diagram of the glycolytic pathway.

Glycolysis occurs through a series of chemical reactions mediated by specific enzymes - phosphoglucose isomerase (PGI); phosphofructokinase (PFK); glyceraldehyde-3-phosphate dehydrogenase (GAPDH); phosphoglycerate kinase (PGK); phosphoglycerate mutase (PGM); pyruvate kinase (PK).

1.4.2 Oxidative phosphorylation

Under aerobic conditions, cells generate ATP through the process of oxidative phosphorylation. Pyruvate generated via glycolysis, is transported to the mitochondria where it enters the tricarboxylic acid (TCA) cycle [188]. The TCA cycle consists of a series of reactions resulting in the oxidation of acetyl CoA to 2 molecules of CO₂ [186]. This generates high energy electrons to support the synthesis of ATP via the electron transport chain (i.e. oxidative phosphorylation). Acetyl CoA is first generated by the oxidative decarboxylation of pyruvate by the pyruvate dehydrogenase complex (Figure 1.8), generating a molecule of CO₂ and NADH [189, 190].

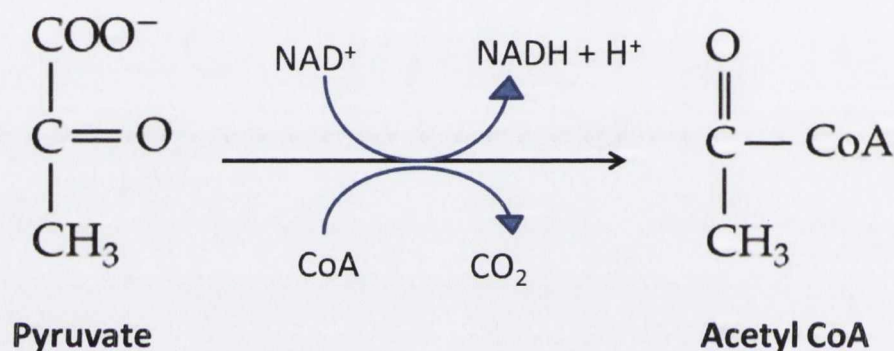


Figure 1.8: Generation of Acetyl CoA from pyruvate

The NADH molecule acts as electron donor to power the electron transport chain (ETC), a series of linked electron carriers which drive ATP synthesis [186, 189]. Both complex I and II, of the ETC, transfer electrons to CoQ from reduced substrates (NADH and succinate respectively). Complex I passes electrons from NADH to CoQ. As electrons are transferred to CoQ, four protons are translocated to the intermembrane space within the mitochondria (Figure 1.9). Complex II passes electrons from succinate (generated in the TCA cycle) to CoQ. Complex III shuttles electrons from CoQ to cytochrome c, again translocating four protons to the intermembrane space [191]. Cytochrome c is responsible for the transfer of electrons to complex IV [192] which catalyses the oxidation of cytochrome c and the reduction of O₂ in the following reaction:



Complex IV also results in the translocation of four further protons into the intermembrane space. This translocation of protons by complex I, III and IV, generates an electrochemical gradient which is used to power ATP synthesis. The influx of protons through complex V (ATP synthase) drives the catalytic subunit to form ATP [193]. Overall the process of oxidative phosphorylation contributes to the production of 36 molecules of ATP per molecule of glucose, making it a far more efficient process in terms of ATP synthesis in comparison to glycolysis alone [186, 188, 193].

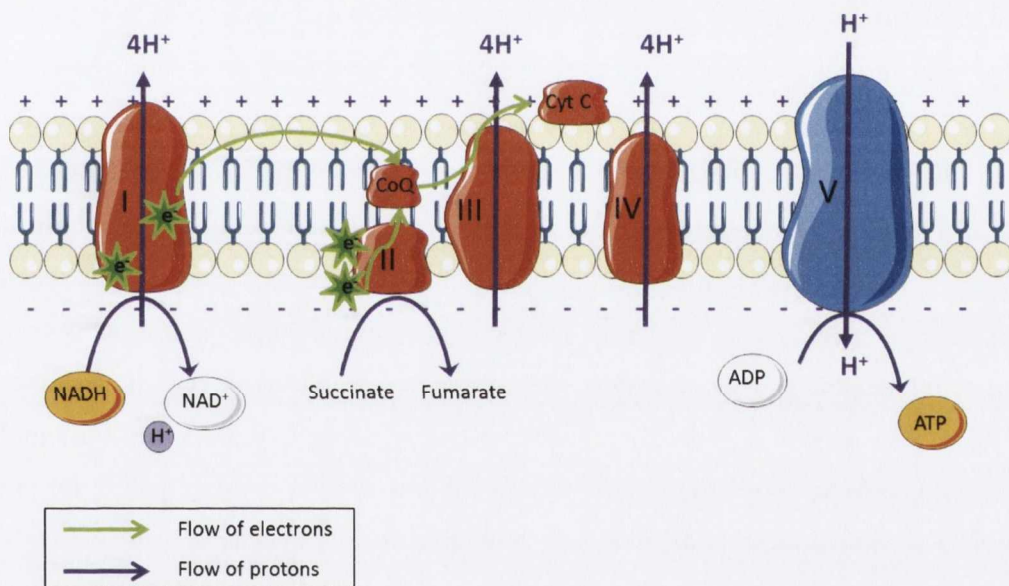


Figure 1.9: Electron flow through the electron transport chain.

1.4.3 The Warburg effect

As mentioned under normoxic conditions, most normal resting cells in the body will rely on oxidative phosphorylation as their primary energy source, generating 36 molecules of ATP per molecule of glucose [186, 188, 193]. However under hypoxic conditions, a cell can undergo anaerobic glycolysis, allowing for the production of 2

molecules of ATP per glucose molecule [186, 187]. Cancer cells however, have been shown to be highly glycolytic, even in the presence of adequate oxygen [194]. This reliance on “aerobic glycolysis” was first described by Otto Warburg and termed “The Warburg effect”. While it is far more inefficient in terms of ATP generation it is a more rapid process than OxPhos, provides NAD^+ to enable glycolysis and supplies metabolic intermediates for lipid, protein and carbohydrate synthesis [186]. Thus it provides distinct advantages for rapidly dividing cells [195]. This increase in glycolysis has been documented in numerous cancer types including breast, gastric, oesophageal squamous cell and lung carcinomas [196]. Studies from our own department have also demonstrated increased glycolysis and OxPhos in Barrett’s and OAC tissue compared to normal oesophageal tissue [37].

1.5 T cell metabolism

Over the course of an immune response, T cells face a number of unique energetic demands. Following antigen recognition and activation, naïve T cells must rapidly grow, divide and acquire a host of effector functions leading to the effective control of the specific pathogen. This requires rapid alterations in nutrient uptake and metabolism with the T cell population. Once the antigenic stimulation/pathogen has been controlled, the vast majority of effector T cells die, however a small pool of memory T cells remain behind to rapidly respond to future challenges. Further metabolic switches must occur in memory T cells to enable their development, endurance and rapid response capabilities. Therefore numerous metabolic switches are required for an appropriate T cell response [197]. Naïve T cells, like normal healthy cells in the body are actively maintained in a quiescent state and rely on oxidative phosphorylation (OxPhos) as their primary source of energy [197-199]. Glucose is taken up by the cells through glucose transporters (GLUT), and is converted to pyruvate via glycolysis. Pyruvate then enters the mitochondria and is decarboxylated to acetyl-CoA, allowing for entry to the mitochondrial TCA cycle and ATP generation [186].

1.5.1 Activated T cells

Upon T cell activation, glucose uptake is increased and pyruvate is diverted away from the mitochondria to be converted into lactate by lactate dehydrogenase (LDH) [198]. This process is termed aerobic glycolysis and has a net production of 2 molecules of ATP per molecule of glucose. As mentioned before while glycolysis produces less ATP than OxPhos, it provides distinct advantages for rapidly dividing cells, by providing

biosynthetic intermediates for lipid, protein and carbohydrate synthesis [186]. Glutamine metabolism is also essential for effector T cell function [200]. Glutamine can independently fuel the TCA cycle in the form of α -ketoglutarate through glutaminolysis, or in the form of citrate through carboxylation [197].

1.5.2 Memory and regulatory T cells

Memory T cells have a far longer life span than the short lived effector cells, and display yet again unique energetic requirements. Like naïve T cells they rely on OxPhos as their primary energy source; however one unique feature is their increased mitochondrial spare respiratory capacity (SRC) allowing for increased ATP generation following re-exposure to its antigen [197, 201, 202]. Regulatory T cells are far less dependent on glucose for survival. In effector T cells, failure to upregulate glycolysis, results in decreased proliferation, reduced cytokine expression and in the presence of antigenic stimulation can lead to T cell anergy [203, 204]. Regulatory T cells however, express low levels of glucose transporters and primarily rely on fatty acid oxidation [205, 206].

1.5.3 Effects of local microenvironment on T cell metabolism and function

The site of inflammation or local tumour environment can represent a harsh microenvironment in which T cells are exposed to numerous stimuli that alter their metabolism and function. These areas tend to be characterised by reduced oxygen and nutrient availability and also by reduced pH, which impose various metabolic stresses upon T cells and alter their function substantially.

Hypoxia

It is clear that oxygen availability can regulate T cell function, differentiation and metabolic state. Hypoxic responses are generally seen at a $PO_2 \leq 1\text{kPa}$ ($\leq 10\text{mmHg}$) [207]. While circulating cells are unlikely to experience hypoxia, many tissues under normal physiological conditions may experience low oxygen levels. For example oxygen readings in the thymus have been shown to be just 10mmHg [208], while the lumen of the GI tract is essentially anoxic (no oxygen), meaning the epithelial cells of the GI tract experience steady state hypoxia [209, 210]. T cells are highly migratory cells meaning they encounter a range of different oxygen tensions; ranging from the low oxygen environment in the thymus, to high oxygen levels in the circulation. Furthermore effector cells at sites of inflammation will be exposed to hypoxic

conditions for prolonged periods [210]. Thus T cells must be able to respond to and function in hypoxic conditions. HIF-1 α is an important transcriptional regulator of the hypoxic response. Under normoxic conditions HIF-1 α is hydroxylated by prolyl hydroxylase (PHD) enzymes, which targets HIF-1 α for degradation. Under hypoxic conditions however, PHDs are inactivated, stabilising HIF-1 α and allowing for its translocation to the nucleus. Here it induces a programme of genes important for cell survival under hypoxic conditions. One of the major functions of HIF-1 α is to induce the metabolic reprogramming of cells to upregulate glycolysis. It results in the upregulation of GLUT1 and glycolytic enzymes such as phosphoglycerate kinase and pyruvate kinase muscle [205, 211]. Furthermore, T cells possess an alternative mechanism for HIF-1 α stabilisation; TCR stimulation results in robust stabilisation of the protein, and can be further enhanced under hypoxic conditions [212, 213]. Hypoxic conditions have been reported to reduce CD4⁺ T cell proliferation and cytokine production [214, 215], however CD8⁺ T cell function is unaffected [216]

Nutrient deprivation

As mentioned cancer cells are known to be highly glycolytic [196] with Barrett's oesophageal and OAC tissue also demonstrating increased levels of glycolytic markers [37]. This increased rate of glycolysis results in a number of changes to the local microenvironment; firstly to fuel this increase, glucose uptake will increase leaving behind a limited availability for infiltrating immune cells. Secondly the increased rate of glycolysis results in the tumour microenvironment having an acidic pH. Reduced availability of glucose in the local environment is associated with immunosuppression. Glucose deprivation has been reported to decrease CD8⁺ T cell effector function, decreasing the production of IFN- γ and cytolytic activity [203, 217]. Increased metabolic stress due to nutrient deprivation can also lead to oxidative stress in T cells [218-220]. Studies have demonstrated that oxidative stress can upregulate the expression of IL-4, IL-5 and IL-13, thus favouring Th2 polarising conditions for T cells [221, 222].

Low pH

The acidic nature of the tumour microenvironment arises due to both the increased production of H⁺ during glycolysis [223] and the increased conversion of pyruvate to lactate [224]. This creates an extracellular pH of around 6.5 compared with normal tissues which are more neutral at pH 7.2-7.4 [225-227]. While an acidic pH of 6.5 can

enhance neutrophil activation, it can exert immunosuppressive effects on T cells [228, 229]. A study by Calcinotto *et al.*, demonstrated that decreasing extracellular pH can lead to a state of anergy in CD8⁺ tumour specific T cells [229]. This state was associated with decreased cytolytic activity, cytokine secretion and reduced TCR expression and signalling. By buffering the pH with administration of esomeprazole, however, these effects could be reversed [229]. This effect has also been demonstrated with excess lactate. Since T cells rely on glycolysis during activation, they also contribute to lactate production. For the continuation of glycolysis however, lactate must be removed from the cells. This relies on export by monocarboxylate transporters-1 (MCT-1) and depends on a gradient between cytoplasmic and extracellular lactate concentrations [230]. Fischer *et al.*, demonstrated that high extracellular lactic acid concentrations block lactate export in T cells, and inhibits T cell proliferation, cytokine production and cytolytic activity [230].

1.5.4 Targeting T cell metabolism

Modulation of T cell metabolism provides a strategy to alter T cell phenotype and function and has been examined in other pathologies. Similar to BO, chronic inflammation in asthma is considered to be Th2 mediated, with increased levels of IL-4, IL-5 and IL-13 demonstrated in patients [231]. Activated CD4⁺ T cells isolated from asthma patients have been shown to overexpress PDK1 (pyruvate dehydrogenase kinase 1) compared to normal patients. This results in the upregulation of glycolysis and the increased production of lactate [232]. Inhibition of glycolysis with DCA (dichloroacetate), inhibited lactate production and resulted in reduced T cell proliferation, and production of IL-5 and IL-17, thus reducing airway inflammation [232]. Thus understanding the metabolic challenges imposed upon T cells and understanding their metabolic profiles in GORD, BO and OAC, may provide vital information to modulate T cell phenotype and function.

Hypothesis and Aims

The overall hypothesis of this study was that factors present in the local oesophageal microenvironment in oesophagitis, BO and OAC could alter T cell phenotype and function and ultimately play a role in disease progression. As such we aimed to carry out an in-depth analysis of T cell activation, chemokine and metabolic profile in the GORD-BO-OAC sequence; examine the composition of the local oesophageal

microenvironment, and assess its impact of T cell profile and function; with the overall aim of identifying novel therapeutic targets.

Specific aims:

- Characterise the T cell phenotype in GORD, BO, OAC tissue
- Assess the impact of oesophageal microenvironment on T cell phenotype and function
- Examine chemokine pathways responsible for T cell recruitment to oesophagitis, BO and OAC tissue
- Examine possible metabolic mechanisms driving alterations in T cell phenotype during disease progression
- Identify potential therapeutic strategies through thorough characterisation of mechanisms altering T cell profile throughout disease progression

2. Chapter 2- Materials and Methods

2.1 Study population and sample acquisition

All patients gave informed consent for use of their samples in this study, which was granted approval by St James' Hospital/AMNCH Ethical Review Board. The study cohort consisted of 58 patients (40 males and 18 females). The mean age of the cohort was 61yrs and ranged from 24 to 81yrs (Table 2.1).

2.1.1 Acquisition of human oesophageal biopsies

Biopsies were obtained from oesophagitis, Barrett's oesophagus (BO) and oesophageal adenocarcinoma (OAC) patients undergoing endoscopy at the outpatient endoscopy clinic at St. James's Hospital. Control (normal) biopsies were taken from either the mid oesophagus of oesophagitis patients or from BO patients from an area clear of metaplasia, and were free from metaplastic tissue as assessed by PCR screen.

2.1.2 Acquisition blood samples

Whole blood samples were collected from oesophagitis, BO and OAC patients undergoing endoscopy at the outpatient endoscopy clinic at St. James's Hospital. OAC bloods were collected prior to any treatment regime. Control bloods were donated from healthy volunteers within the department.

2.2 Processing of blood samples

2.2.1 Serum collection

Serum samples were prepared from blood collected in serum clot activator tubes (Grenier Bio One, Frickenhausen, Germany). Blood was allowed to clot at room temperature for 30min. Following centrifugation at 3,000rpm for 10 min at 4°C, serum layer was removed and stored at -80°C until use.

2.2.2 PBMC isolation

Whole blood was collected in EDTA tubes (Grenier Bio One, Frickenhausen, Germany) and peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation using Ficoll-Paque TM Plus (GE Healthcare, Uppsala, Sweden). Blood samples were diluted in an equal volume of phosphate buffered saline (PBS) solution and were layered on Ficoll medium (to a final ratio of 2:3 Ficoll: blood). Cells were spun at 400 x rcf for 30min at room temperature with the centrifuge break off so as not

to disrupt the resulting layers. The intermediate PBMC buffy layer was then removed and washed with PBS. Cells were resuspended in PBS and counted using trypan blue (1:10). Cells were then pelleted by centrifugation and resuspended in RPMI supplemented with 10% (v/v) foetal calf serum (FCS) and 1% (v/v) pen-strep (50U/ml penicillin and 100µg/ml streptomycin).

2.3 Generation of tissue conditioned media

Biopsies were cultured in 1ml X-VIVO™ 15 (Lonza) media for 24hr at 37°C and 5% CO₂. X-VIVO™ 15 is a serum free medium specially formulated to support the proliferation of CD3+ T cells from peripheral blood and tumour. It was chosen to circumvent the need for foetal calf serum in the culture medium. Tissue conditioned media (CM) was then collected and stored at -80°C, biopsies were snap frozen in liquid nitrogen and stored at -80°C for subsequent analysis.

2.4 RT-PCR and protein quantification

In order to verify that the BO biopsies contained metaplastic tissue, each biopsy was assessed for villin expression. Villin is an end-differentiated marker of intestinal cells, and the protein needs to be of a sufficient quantity to result in a mature brush border and has been shown to be present in BO tissue [38].

2.4.1 RNA and protein isolation

RNA and protein were isolated from biopsies using Qiagen AllPrep RNA/Protein Kit (Qiagen, Limburg, Netherlands). Biopsies were incubated in 200µl buffer APL for 5min and were homogenised using a tissue lyser at 25Hz for 5min and a 5mm stainless steel bead. RNA and protein were then isolated as per product protocol. The BCA (bicinchoninic acid) Protein Assay (Pierce, Thermo Scientific, IL, USA) was used to determine protein concentration in each biopsy.

2.4.2 Protein quantification

The BCA (bicinchoninic acid) Protein Assay (Pierce, Thermo Scientific, IL, USA) was used to determine protein concentration in biopsies. A standard curve, covering a range of concentrations from 0µg/ml to 2000µg/ml was generated using a stock solution of BSA (2mg/ml). 25µl of each standard or sample was added, in duplicate, to a well in a 96-well plate. 200µl working reagent (50:1, Reagent A:B) was added to each well and

Table 2.1: Patient demographics according to disease stage

	Normal*	Oes	BO	OAC
# patients	25	10	23	31
Mean age (range)	59.3 (24-81)	52.3 (33-67)	59.7 (24-81)	64.0 (45-79)
% Males	75.0	50.0	73.9	64.5
Oesophagitis grade				
Grade a	-	7	-	-
Grade b	-	1	-	-
Grade c	-	2	-	-
% Patients on PPIs	-	50	-	-
Length of Barrett's				
3-5cm	-	-	13	-
>5cm	-	-	10	-
% Patients on PPIs	-	-	100	-
Tumour category				
T1	-	-	-	2
T2	-	-	-	1
T3	-	-	-	22
T4	-	-	-	1
Not known	-	-	-	5
Nodal status				
N0	-	-	-	11
N1	-	-	-	12
N2	-	-	-	4
N3	-	-	-	1
Not known	-	-	-	3

*Normal group made up of patients from Oesophagitis and Barrett's group, tissue taken from an area free from metaplasia or inflammation. – indicates that data was not applicable to specified group.

mixed on a plate shaker for 30sec. Following 30min incubation at 37°C, samples were allowed to cool to room temperature and absorbance was read at 562nm (Molecular Devices, Versamax microplate reader) and analysed using Softmax Pro 5.4.1 software.

2.4.3 RNA quantification and purity analysis

RNA concentration was determined spectrophotometrically using a MaestroNano (Maestrogen, NV, USA). Instrument was blanked prior to sample reading using 1µl RNase free water. 1µl of each sample was loaded onto the instrument and the concentration was measured in ng/µl. Purity of RNA was assessed using the 260:280 ratios with a ratio greater than 1.65 indicative of a relatively pure RNA yield. A 260:230 ratio of greater than 1.7 indicated that the sample was free of phenol contamination.

2.4.4 cDNA synthesis

cDNA was synthesised by adding 1µl random primers (0.5µg/µl) to a total volume of 11µl containing 60ng RNA in RNase free water. Following heating at 70°C for 10min to denature RNA, sample was then placed on ice for 1min. A master mix containing the following was added to each sample: RNaseOUT recombinant ribonuclease inhibitor (1Unit/µl), dNTPs (10mM, prepared as a 1:1:1:1 ratio of dATP, dGTP, dTTP and dCTP), Bioscript reverse transcriptase (200Unit/µl) in 5X Bioscript reaction buffer. The mixture was then incubated at 37°C for 1hr and 70°C for 10min. The resulting cDNA was stored at -20°C until use. All products were purchased from Invitrogen (Life technologies, CA, USA) except for Bioscript which was from Bioline (London, UK).

2.4.5 Reverse transcription polymerase chain reaction (RT-PCR)

1µl cDNA was used as a template for PCR. Samples were prepared using Mangomix Master mix (Bioline, London, UK), forward primer (5µM), reverse primer (5µM) and RNase free water as follows (Table 2.3). Template cDNA was initially denatured at 95°C for 5min. Each cycle consisted of primer denaturation (94°C for 30sec), annealing (58-62°C for 30sec) and extension (72°C for 1min) for 38 cycles. This was followed by a final elongation step of 72°C for 5min. All primers were purchased from sigma (Sigma Aldrich, MO, USA) and rehydrated to a stock concentration of 100µM in RNase free water, and diluted to a working concentration of 20µM before use. Primer sequences and PCR conditions are outlined below (Table 2.4).

Table 2.2: Composition of samples for cDNA synthesis

	Volume (μl) per sample
Random primers (0.5μg/μl)	1
60ng RNA + RNase free H2O	11
Heat at 70°C for 10min, cool on ice for 1min, then add to the following:	
5X Bioscript reaction buffer	4
RNase free H2O	2
DNTPs	1
RNaseOUT	0.5
Bioscript Reverse Transcriptase	0.5
Total Volume	20

Table 2.3: Composition of samples for PCR

	Volume (μl) per sample
Mangomix Master Mix	10
Forward primer	1
Reverse primer	1
RNase free H2O	7
cDNA	2
Total volume	21

Table 2.4: Primer sequences

Gene	Primer sequence	Annealing temp (°C)
β-actin	Fw 5'-TGAGAGGGAAATCGTGCGTG-3'	58
	Rev 5'-TGCTTGCTGATCCACATCTGC-3'	
Villin	Fw 5'-AATGGCCACCATGGAGAACA-3'	62
	Rev 5'-ACCACAATTCTGTCTTTCACGG-3'	

2.4.6 Agarose gel electrophoresis

PCR products were visualised by electrophoresis through 1.4% agarose. The agarose solution was prepared in 1X Tris acetate EDTA (TAE) buffer and heated in a microwave for 90sec to allow the agarose to dissolve. TAE buffer consists of 40mM Tris base, 20mM glacial acetic acid in 1mM EDTA. Solution was allowed to cool before the addition of ethidium bromide (10µl in 100ml agarose solution). The solution was then poured into a gel tray with a comb depth of 3-5mm and allowed to set for approx 20-30min. The gel was then covered with 1X TAE buffer and combs were removed. Samples were loaded into the gel and run at 100V in a EC-360 Maxicell gel system. The bands were visualised and photographed under UV light (Figure 2.1). β-actin was used as an endogenous control. If normal or oesophagitis biopsies expressed villin, or if BO or OAC biopsies lacked villin expression, they were excluded from study.

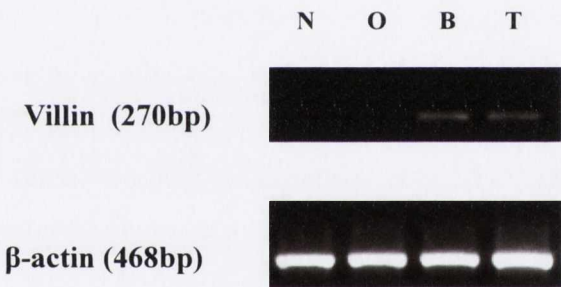


Figure 2.1: Examples of PCR for validation of patient biopsies.

Villin was used as a marker for intestinal metaplasia and thus was used to confirm presence of BO and OAC tissue. N= Normal, O=Oesophagitis, B=Barrett’s, T= oesophageal adenocarcinoma biopsy. B-actin was used as a control.

2.5 Tissue digestion

Biopsies were enzymatically digested to allow for T cell phenotyping. Biopsies were washed briefly in wash buffer (Krebs ringer solution, 1% (w/v) BSA). Krebs ringer was comprised of: 118mM NaCl, 24.8mM NaHCO3, 1.2mM KH2PO4, 4.8mM KCl, 1.25mM CaCl2 (Fluka), 1.2mM MgSO4 and 10mM HEPES. Tissue was teased apart using a scalpel and placed in a 15ml tube containing collagenase buffer (Krebs ringer

buffer, 4% (w/v) BSA, 2mg/ml collagenase type IV). Following 25min incubation at 37°C and 200rpm on an orbital shaker (Stuart, mini orbital shaker), the resulting dissociated tissue was strained through a 70µm filter and washed in wash buffer by centrifugation at 360 x rcf for 5min. Cells were then re-suspended in X-VIVO™ 15 media (Lonza) and incubated at 37°C and 5% CO₂ prior to staining for flow cytometry. Except were indicated, all reagents were purchased from Sigma (Sigma Aldrich, MO, USA).

2.6 Cell lines and cell culture

All cell culture media was purchased from Lonza (Basel, Switzerland) and all plastics were purchased from Starstedt Ltd. (Wexford, Ireland) unless otherwise stated. Cell culture was carried out in a dedicated cell culture room in a grade II laminar flow hood using aseptic technique. Het-1A, QH, GO and OE33 cell lines, representing normal squamous oesophageal, Barrett's metaplasia, High grade dysplasia and oesophageal adenocarcinoma (Figure 2.2) were used in this study. Het-1A, QH and GO cell lines were obtained from American Type Culture Collection (ATCC) (LGC Standards, Middlesex, UK). The OE33 cell line was sourced from the European Collection of Cell Cultures (Salisbury, UK). Het-1A cells were maintained in antibiotic-free bronchial epithelial cell basal media (BEBM) enhanced with hormonal cocktail BEGM single quotes (Lonza, Basel, Switzerland). These include Bovine Pituitary Extract (BPE), Hydrocortisone, human Epidermal Growth Factor (hEGF), Epinephrine, Transferrin, Insulin, Retinoic Acid, and Triiodothyronine. QH and GO cell lines were maintained in BEBM media enhanced with BEGM single quotes as above with the addition of Gentamicin/Amphotericin-B, and supplemented with 10% FCS (foetal calf serum) and 1% pen-strep (50U/ml penicillin and 100µg/ml streptomycin) (Lonza). The OE33 cell line was maintained in Roswell Park Memorial Institute (RPMI) 1640 with L-glutamine media (Lonza) supplemented with 10% FCS and 1% pen-strep. Cells were passaged when they reached 70-80% confluency and were incubated at 37°C and 5%CO₂.

2.6.1 Cell subculture

Cells were examined daily using an inverted phase microscope (Nikon corporation, Tokyo, Japan) and were subcultured upon reaching 70-80% confluency. Growth media was removed and cells were washed with PBS. 1ml of trypsin ethylene diamine tetra acetic acid (EDTA) was added to the flask and incubated at 37°C for 3min until cells

had detached. 10ml of complete media was then added to the flask to deactivate the trypsin and cells were pelleted by centrifugation at 1300rpm for 3min. Cells were resuspended in fresh media and seeded into new flasks.

2.6.2 Generation of cell line supernatant

To assess the effect of cell line supernatant on T cell phenotype and function, cells were grown for 4 days to 80% confluency, media was then removed prior to trypsinisation, centrifuged at 1300rpm to remove any cell debris and stored at -80°C until use

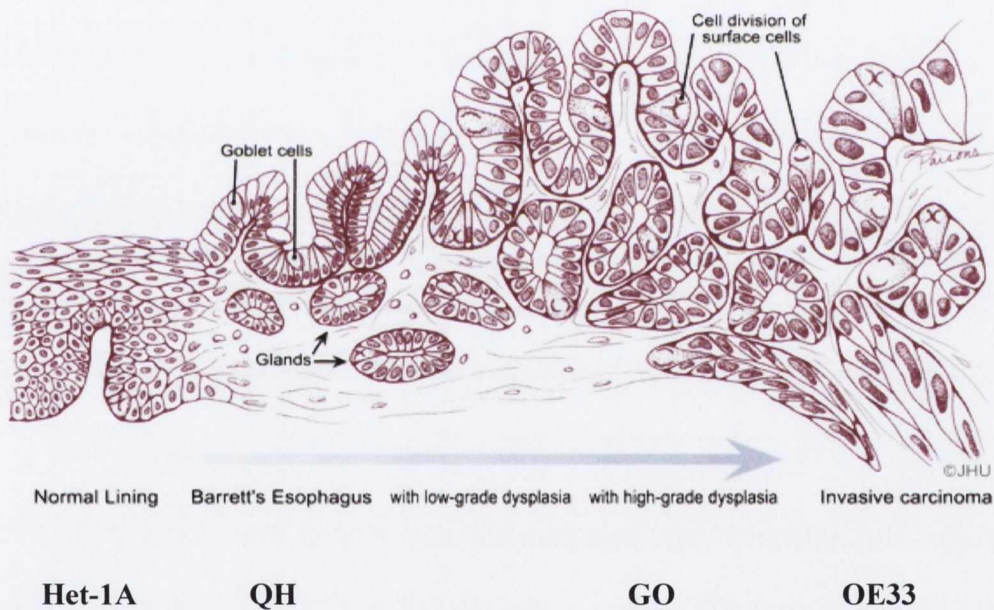


Figure 2.2: Barrett's oesophagus cell line sequence.
Het-1A cells represent healthy oesophageal squamous epithelium. QH and GO cells are derived from Barrett's oesophagus with no dysplasia and high grade dysplasia respectively. OE33 cells were derived from invasive Barrett's oesophageal adenocarcinoma. (Image adapted from <http://pathology2.jhu.edu/beweb/definition.cfm>).

2.6.3 Mycoplasma testing

Cell lines were regularly tested for mycoplasma infection. Supernatant from a confluent flask of cells was collected and stored at 4°C until use. Supernatant was spun at 2000rpm to pellet any cellular debris. 1µl cell line supernatant was used as a template for PCR. Samples were prepared using Mangomix master mix (Bioline, London, UK), forward primer (5'-GGGAGCAAACAGGATTAGATACCCT-3') and reverse primer (5'-TGCACCATCTGTCACTCTGTTAACCTC-3') and dH₂O (AccuGENE, Lonza) as per Table 2.5. Samples were initially denatures at 95 °C for 5 min. Each cycle consisted of primer denaturation (94 °C for 30sec), annealing (55 °C for 30sec) and extension (72 °C for 1 min) for 40 cycles. This was followed by a final elongation step of (72 °C for 10min). Samples were then visualized by electrophoresis as per section 2.4.6.

Table 2.5: Composition of samples for mycoplasma PCR

	Volume per sample (µl)
Mangomix Master Mix	12.5
Forward primer (10µM)	0.5
Reverse primer (10µM)	0.5
AccuGENE water	10.5
Cell line supernatant	1
Total Volume	25

2.7 Treatment of PBMC culture

2.7.1 Treatment of PBMC with conditioned media

To assess the effect of TCM on T cell phenotype and function, PBMC were isolated as described and seeded at a density of 5 x 10⁵ cells per well in a 24 well plate. TCM (500µl) was added to wells in a 1:1 ratio, control wells were treated with X-vivo media only. Cells were incubated at 37°C and 5% CO₂ for 24hr and examined by flow cytometry for T cell activation and functional status.

2.7.2 Treatment of PBMC with cell line supernatant

PBMC were isolated as described and seeded at a density of 1 x 10⁶ cells per well in a 12 well plate. Cell line supernatant was added to wells in a 1:1 ratio, control wells were treated with the respective media only (BEBM, or RPMI). Cells were incubated at

37°C and 5% CO₂ for 24hr and examined by flow cytometry for T cell activation and functional status.

2.7.3 Culture of PBMC under glucose deprivation

To assess the effect of glucose deprivation on T cell phenotype and function, PBMC were isolated as described and seeded at a density of 5×10^5 cells per well in a 24 well plate in RPMI +/- glucose. Glucose free media was purchased from Invitrogen (Life technologies, CA, USA), complete RPMI was from Lonza (Basel, Switzerland) and contained 11mM glucose. Cells were incubated at 37°C and 5% CO₂ for 24hr and examined by flow cytometry for T cell activation, cytokine expression and metabolic profile.

2.7.4 Culture of PMBC under low pH conditions

To assess the impact of an acidic microenvironment on T cell phenotype and function, PBMC were isolated as described and seeded at a density of 5×10^5 cells per well in a 24 well plate in RPMI at normal (7.0) or low pH (6.5). Hydrochloric acid (Sigma, MO, USA) was used to reduce the pH of complete RPMI to 6.5. Cells were incubated at 37°C and 5% CO₂ for 24hr and examined by flow cytometry for T cell activation, cytokine expression and metabolic profile.

2.7.5 Inhibition of Oxidative phosphorylation with rotenone

To assess the impact of inhibiting oxidative phosphorylation on T cell phenotype and function, PBMC were isolated as described and seeded at a density of 5×10^5 cells per well in a 24 well plate. Cells were treated with 1µM rotenone and incubated at 37°C and 5% CO₂ for 24hr. Cells were then examined by flow cytometry for T cell activation, cytokine expression and metabolic profile.

2.8 Flow cytometry

2.8.1 Flow cytometry analysis of surface activation markers

PBMC were washed (1300rpm, 3min) with FACS buffer (PBS containing 2% FCS and 0.1% Sodium azide) and resuspended in 1ml blocking buffer (FACS buffer containing 50% FCS). Following 5min incubation at room temperature cells were again washed in FACS buffer and incubated with appropriate surface antibodies (see Table 2.6) for 30 min at 4°C protected from light. Cells were then washed twice and resuspended in

500µl of FACS buffer and analysed by flow cytometry. Data was acquired on a CyAn™ ADP Analyzer (Beckman Coulter) using Summit v4.1 and analysed using FlowJo v7.6.1. Gating strategy used is shown in Figure 2.3. FMO (fluorescence minus one) controls were used in all experiments to identify gates and account for spectral overlap.

2.8.2 Intracellular cytokines

In order to detect levels of intracellular cytokines, PBMC were stimulated with PMA (10ng/ml) (Sigma, MO, USA) and ionomycin (1µg/ml) (Sigma, MO, USA) for 1hr at 37 °C and 5% CO₂. Brefeldin A (10µg/ml) (Sigma, MO, USA) was then added for a further 3hr. Cells were then treated as above (section 2.8.1). Briefly cells were washed with FACS buffer, resuspended in blocking buffer and incubated for 5 min at room temperature. Cells were again washed in FACS buffer and incubated with surface antibodies (see Table 2.7) for 30 min at 4°C protected from light. Cells were then washed twice in FACS buffer and fixed with 50µl of fixation solution A (Fix and Perm cell permeabilization kit, Caltag Laboratories, Buckingham, UK) for 15 min at room temperature. Following washing with FACS buffer, cells were treated with 50µl permeabilization solution B (Fix and Perm cell permeabilization kit, Caltag Laboratories, Buckingham, UK) and appropriate intracellular antibodies (see Table 2.7) and incubated for 15 min at room temperature. Cells were then washed and resuspended in 500µl FACS buffer and analysed immediately by flow cytometry. Data was acquired on a CyAn™ ADP Analyzer (Beckman Coulter) using Summit v4.1 and was analysed using FlowJo v7.6.1. Gating strategy used is demonstrated in Figure 2.4. As PMA downregulates human CD4, cells were gated on based on CD3 and CD8 staining only. CD8 cells were CD3⁺CD8⁺ were CD4 cells were represented by CD3⁺CD8⁻. While this population may include a small proportion of innate lymphocytes, the majority of the population is made up of CD4⁺ T cells.

Table 2.6: FACS antibodies for surface markers

Surface Marker	Fluorochrome	Clone	Company	Volume (µl)
CD3	APC-Cy7	HIT3a	Biolegend	1
CD4	APC	SK3	Biolegend	1.5
CD8	Brilliant violet	RPA-T8	BD bioscience	0.5
CD69	PE	FN50	BD bioscience	1
CD45 RA	V500	HI100	BD bioscience	2
CD45RO	FITC	UCHL1	Immunotools	2
CD62L	PeCy7	DREG56	eBioscience	0.5

Table 2.7: FACS antibodies for intracellular cytokines

Surface antibodies	Fluorochrome	Clone	Company	Volume (µl)
CD3	PE-eFluor 610	UCHT1	eBioscience	1
CD8	Brilliant violet	RPA-T8	BD bioscience	0.5

Intracellular Abs	Fluorochrome	Clone	Company	Volume (µl)
IL-12	FITC	C11.5	BD bioscience	1
IL-4	PE	8D4-8	BD bioscience	1
TNF- α	PerCP-Cy5.5	Mab11	Biolegend	2
TGF- β	Pe-Cy7	TW4-2F8	Biolegend	2
IFN- γ	V500	B27	BD bioscience	2
IL-10	APC	JES3-19F1	BD bioscience	2
IL-17	APC-Cy7	BL168	Biolegend	2

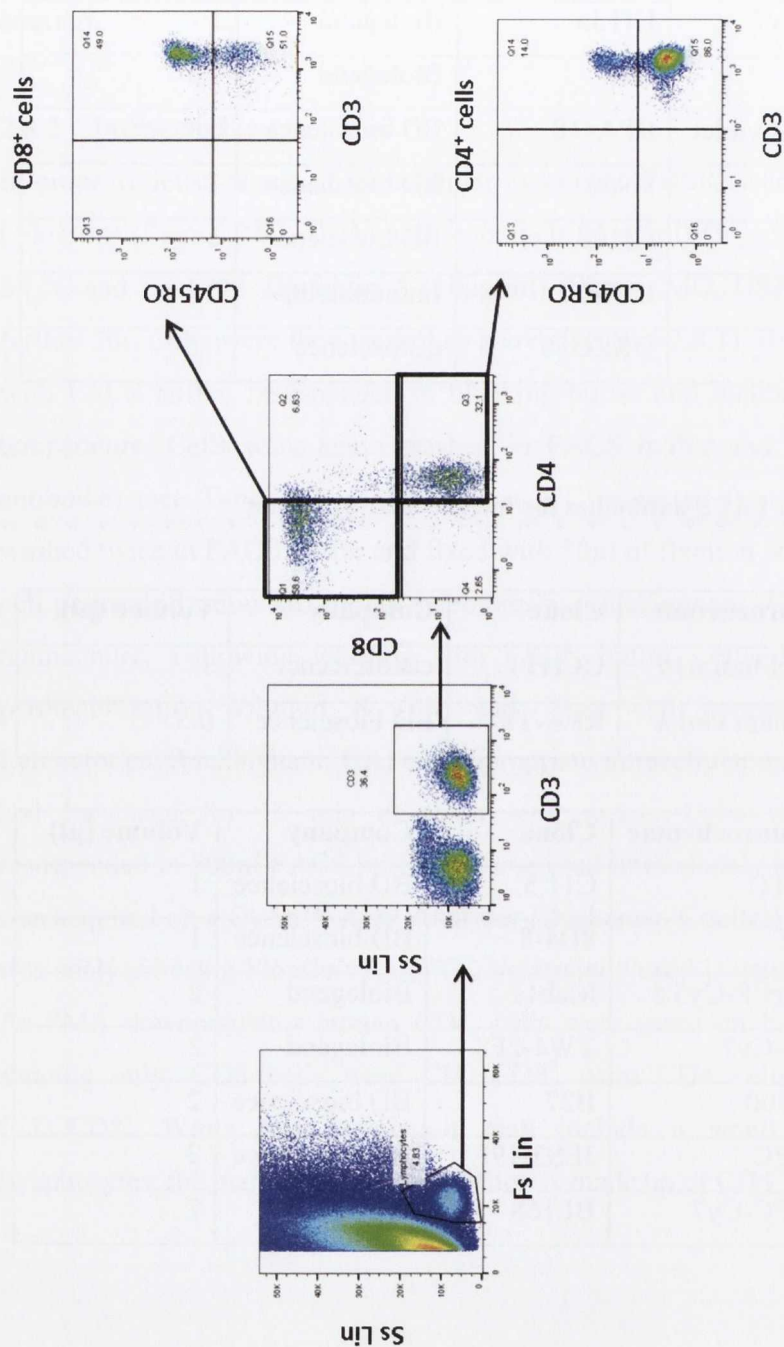


Figure 2.3: Flow plots show gating strategy used for analysis of surface staining.

Lymphocytes were first gated on based on forward scatter and side scatter and CD3⁺ T cells were then identified. CD4⁺ and CD8⁺ T cells were identified allowing for the quantification of CD4 (CD3⁺CD4⁺CD8⁻), and CD8 (CD3⁺CD4⁻CD8⁺) cells positive for individual surface markers.

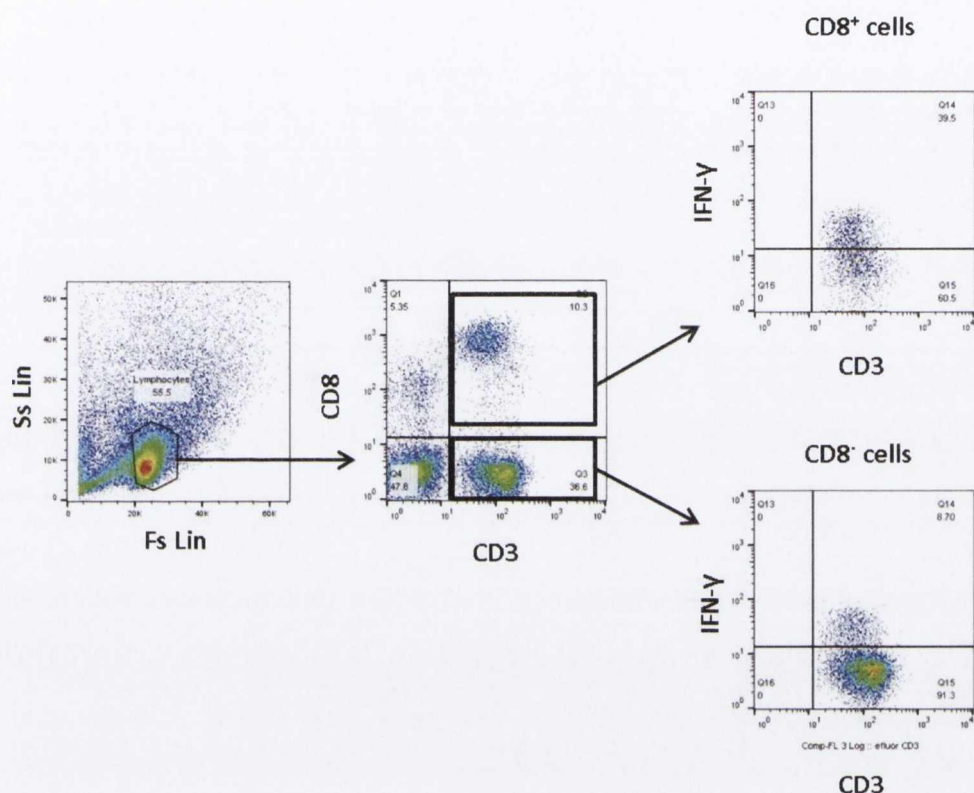


Figure 2.4: Flow plots demonstrating gating strategy for intracellular cytokine staining.

For assays involving stimulation, cells were gated based on CD3 and CD8 as PMA downregulates human CD4. CD8 cells were $CD3^+CD8^+$ and CD4 cells were $CD3^+CD8^-$. Lymphocytes were first gated on based on forward scatter and side scatter. CD4 ($CD3^+CD8^-$) and CD8 ($CD3^+CD8^+$) T cells were identified based on CD3 and CD8 staining allowing for the quantification of CD4 and CD8 cells positive for individual intracellular markers.

2.8.3 Chemokine receptors

To assess T cell chemokine receptor expression in patient blood, whole blood staining was performed. Whole blood staining was used as OAC blood was in limited supply. Appropriate antibodies (table 2.8) were added to 100µl whole blood and incubated for 15min protected from light. Red blood cells were then lysed by the addition of 0.87% ammonium chloride. Cells were incubated at room temperature for 2min and washed with PBS. Cells were then resuspended in 500µl FACS buffer and analysed by flow cytometry. Data was acquired on a CyAn™ ADP Analyzer (Beckman Coulter) using Summit v4.1 and was analysed using FlowJo v7.6.1. Gating strategy used is shown in Figure 2.3.

2.8.4 Metabolism markers

Cells were stained with appropriate surface antibodies (Table 2.9) as previously described (section 2.8.1) for 30 min at 4°C protected from light. Cells were then fixed and permeabilised (as per section 2.8.2) and incubated with appropriate intracellular antibodies (see Table 2.9) for 15 min at room temperature. Cells were then washed and resuspended in 500µl FACS buffer and analysed by flow cytometry. For metabolic studies T cells were identified based on CD3 staining only.

2.9 Proliferation assay

The effect of CLS and CM on proliferative capacity of T cells was examined by CFSE staining. PBMC were isolated as before and stained with CFSE as previously described [38]. Briefly, 1×10^6 cells were thoroughly resuspended in 1ml PBS containing 5% FBS. 110µl PBS was added to the non-wetted portion of tube, ensuring it did not mix with cell suspension, to this 1.1µl of 5mM CFSE was added. Tube was capped and vortexed quickly. Final concentration of CFSE was 5µM. Following 5min incubation at room temperature protected from light, cells were washed 3 times with PBS by centrifugation at 300 x g for 5min at room temp. Pellet was then resuspended in RPMI supplemented with 10% FBS and 1% Pen-Strep and cells were seeded at a density of 2.5×10^5 cells per well and were treated with either CLS or CM at a ratio of 1:1. PBMC were then activated by the addition of low dose PMA (1ng/ml) and ionomycin (0.1µg/ml) and were incubated for 5 days at 37°C 5% CO₂ and analysed by flow cytometry.

Table 2.8: FACS antibodies for chemokine receptor expression

Surface Marker	Fluorochrome	Clone	Company	Volume (µl)
CD3	Pe-eFluor 610	UCHT1	eBioScience	1
CD4	PeCy5	SK3	BD bioscience	1.5
CD8	Brilliant violet	RPA-T8	BD bioscience	0.5
CCR5	FITC	2D7/CCR5	BD bioscience	2
CXCR3	PE	REA232	Miltenyi	2
CCR4	PeCy7	L291H4	Biolegend	2
CCR6	BV510	11A9	BD bioscience	1
CCR3	APC-Cy7	5E8	Biolegend	1

Table 2.9: FACS antibodies for metabolism markers

Surface Abs	Fluorochrome	Clone	Company	Volume (µl)
CD3	PE-eFluor 610	UCHT1	eBioscience	1
GLUT1	Alexa647	Polyclonal	Stratech	1

Intracellular Abs	Fluorochrome	Clone	Company	Volume (µl)
GAPDH	FITC	6C5	Merck Millipore	1
HSP60	Pe	LK2	Abcam	1
ATP5B	PeCy5	Polyclonal	Stratech	1

2.10 ELISAs

2.10.1 Measuring inflammatory cytokine levels

Levels of IFN- γ , TNF- α , IL-1 β , IL-12p70, IL-2, IL-15, GM-CSF, IL-10, IL-6, and IL4 in the cell line supernatant and tissue conditioned media were assessed using MesoScale Discovery multiplex and singleplex ELISAs as per product protocol. Briefly, standards were prepared through 4-fold serial dilutions of stock calibrators. 50 μ l of sample was added per well, plate was sealed and incubated at room temperature with shaking for 2hr. The plate was then washed three times with wash buffer (PBS with 0.05% Tween-20). 25 μ l detection antibody solution was then added to each well and incubated at room temperature with shaking for 2hr. Plate was again washed with wash buffer and 150 μ l read buffer was added to each well prior to analysis. Plates were read using the MesoScale Diagnostics SECTOR imager 2400. Cytokine levels (pg/ml) in tissue conditioned media were normalised to total protein content in biopsy and were expressed as pg/ μ g.

2.10.2 Measuring chemokine levels

Chemokine levels in cell line supernatant, tissue conditioned media and patient serum were measured using MesoScale Discovery multiplex and singleplex ELISAs as per product protocol (see section 2.10.1). Levels of CXCL10, CXCL11, CCL3, MIP-1 β , CCL5, CX3CL1, CCL17, CCL22, CCL2, CCL13, CCL20 and CXCL8 were assessed. Plates were read using the MesoScale Diagnostics SECTOR imager 2400. Chemokine levels (pg/ml) in tissue conditioned media were normalised to total protein content in biopsy and were expressed as pg/ μ g.

2.10.3 Lactate assay

Lactate levels in tissue conditioned media (CM) were measured using a lactate assay kit (Sigma-Aldrich, MO, USA). Samples were filtered through a 10kDa cut-off spin filter (Sigma-Aldrich) to remove high levels of lactate dehydrogenase prior to analysis. Standard curve, covering a range from 0-10 nmole/well was generated as per product protocol. Samples were diluted 1:20 prior to assay. 1 μ l of diluted sample was added to each well and it was brought to a final volume of 50 μ l with the master reaction mix (46 μ l lactate assay buffer, 2 μ l lactate enzyme mix and 2 μ l lactate probe). Samples were then incubated at room temperature for 30min protected from light with shaking. Absorbance was read at 570nm (Molecular Devices, Versamax microplate reader) and

analysed using Softmax Pro 5.4.1 software. Lactate levels were calculated as ng/ μ l and were normalised to total protein content in biopsy and were expressed as ng/ μ g.

2.10.4 Glucose assay

Glucose levels in tissue conditioned media (CM) were measured using a glucose assay kit (Abcam, Cambridge, UK). Samples were deproteinised through 10kDa cut-off spin filter (Sigma-Aldrich). Glucose standard (1nmol/ μ l) was used to generate a standard curve covering a range of concentrations from 0-10nmol/well as per product protocol. Samples were diluted 1:20 prior to assay. 2 μ l of diluted sample was added to each well and was brought to a final volume of 50 μ l with Glucose assay buffer. 50 μ l of glucose reaction mix (46 μ l glucose assay buffer, 2 μ l glucose probe and 2 μ l glucose enzyme mix) was added to each well. Samples were incubated at 37°C for 30min protected from light with shaking. Absorbance was read at 570nm (Molecular Devices, Versamax microplate reader) and analysed using Softmax Pro 5.4.1 software. Glucose levels were calculated as ng/ μ l and were normalised to total protein content in biopsy and were expressed as ng/ μ g.

2.11 Chemotaxis assay

2.11.1 Recombinant chemokines and receptor blockade

Recombinant chemokines were diluted to a working concentration of 1 μ g/ml and were used at a final concentration of 10ng/ml in M199 media for migration studies. All chemokines were purchased from Cell guidance systems (Cambridge, UK) except for CCL3 which was purchased from Biolegend (CA, USA). For blockade experiments PBMC were pretreated with chemokine receptor antagonists for 1hr at 37°C at the following concentrations: 100nM AMG 487 (CXCR3 antagonist) (Bio-technie, Oxford, UK), 10nM Vicriviroc Malate (CCR5 antagonist) (Merckmillipore, Nottingham, UK), 140nM C 021 dihydrochloride (CCR4 antagonist) (R&D systems, Oxford UK), 50nM SB 328437 (CCR3 antagonist) (Tocris, Oxford, UK) 50nM, or SCH 527123 (CXCR1/CXCR2 antagonist) (Stratech, Cambridge, UK).

2.11.2 Chemotaxis assay

Chemotaxis in response to recombinant chemokines was measured using Corning Transwell® plates (6.5mm diameter, 5 μ m pore size, Corning, NY, USA). The bottom well of the transwell plate was loaded with 600 μ l M199 media alone or 10ng/ml

recombinant chemokine, 20% FBS was used as a positive control. The top chamber of the transwell was loaded with 2×10^5 cells in 100 μ l M199 media. Following 2hr incubation at 37°C and 5%CO₂, inserts were removed and cells which had migrated to the bottom chamber were removed and stained for analysis by flow cytometry. Cells were counted using CountBright™ absolute counting beads (Life technologies, Dublin, Ireland). The following equation was used for calculating cell number:

Calculation of cell/ μ L =

$$\frac{A \times C}{B \times D}$$

Where:
A = number of cell events
B = number of bead events
C = number of beads added (lot specific)
D = volume of sample (μ L)

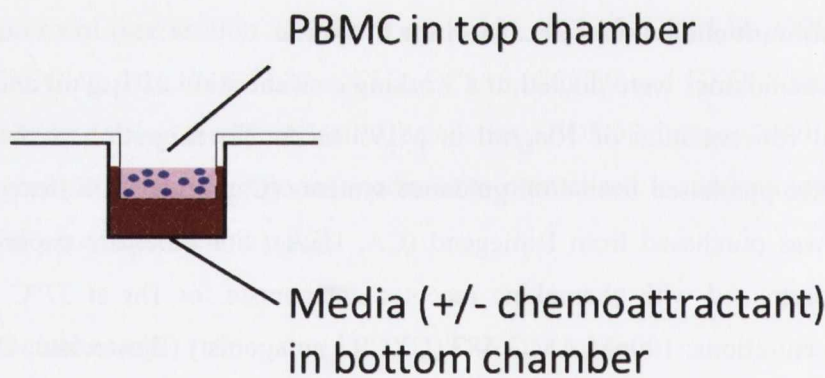


Figure 2.5: Transwell set up for chemotaxis assay.

2.12 Nuclear magnetic resonance spectroscopy

Quantities of metabolites in tissue conditioned media were assessed by NMR spectroscopy carried out by Dr. Lorraine Brennan, Conway Institute, UCD. Tissue conditioned media (300 μ L) were prepared by the addition of 250 μ L deuterium oxide

and 10 μ L sodium trimethyl propionate (TSP) (0.005 g/mL). ^1H NMR spectra were acquired on a 600-MHz Varian NMR spectrometer (Varian Limited, Oxford, United Kingdom) by using the first increment of a NOESY (Nuclear overhauser effect spectroscopy) pulse sequence at 25°C. Spectra were acquired with 16384 data points and 512 scans. Water suppression was achieved during the relaxation delay (2.5s) and the mixing time (100ms). All spectra were referenced to TSP at 0.0 ppm. ^1H NMR ACM spectra were processed manually with Chenomx software (version 7.5; Chenomx Edmonton, Canada) and were phase and baseline corrected. Spectra were converted into 8000 spectral regions of 0.001ppm width. The water region was excluded (4-5.5ppm), and data were normalized to the Total area of the spectral integral. Discriminating metabolites were identified by using libraries of pure metabolites enveloped in house and the Chenomx database library. Relative quantity of the metabolites was achieved using Chenomx.

2.13 Statistical analysis

Data was analysed using Graph Pad Prism (Graph Pad Prism, San Diego, CA, USA) software and was expressed as mean $\pm$ s.e.m. Statistical differences between two groups were analysed using a Student's t-test. For cell line data parametric t-tests were used. Non parametric t-tests (Mann Whitney) were used for patient samples as data was not normally distributed. Statistical differences in mean values between more than two experimental groups were determined by One-way ANOVA. Cell line data used parametric test, whereas non parametric tests (Kruskal Wallis) were again used for patient data. Statistical significance was determined as $p \leq 0.05$. * denotes $p \leq 0.05$, ** denotes $p \leq 0.01$, *** denotes $p \leq 0.001$.

3. Chapter 3- Results

Phenotypical characterisation of T cells in GORD, BO and OAC and determination of the impact of the local microenvironment on T cell profile and function

3.1 Introduction

It was initially proposed that cellular damage that occurs in GORD was a direct result of caustic injury at the luminal surface of the oesophagus due to chronic reflux [70, 71]. However, more recent work has suggested that inflammation is the trigger, with an altered immune microenvironment providing a driving force for disease progression [28, 72, 113, 233]. Souza *et al.*, have alluded to the importance of the inflammatory microenvironment by demonstrating that refluxed acid did not directly damage the epithelial layer of the oesophagus, but instead induced the production of chemokines and cytokines including CXCL8 and IL-1 β , which significantly influence and alter lymphocyte migration to oesophageal tissue [72]. IL-4 has also been identified as a potential modulator of BO, potentially driving the metaplastic transformation when present in the local microenvironment [113]. In a primary cell model, Shan *et al.* showed that IL-4 leads to the down regulation of squamous cell markers, involucrin and keratin 13, while up regulating columnar markers, keratin 7 and keratin 8 and thus IL-4 may play a role in triggering intestinal metaplasia in BO [113]. Furthermore, genetic polymorphisms predisposing patients to a strong pro-inflammatory host response has been associated with increased risk of BO [234]. This implies that the local inflammatory response in the oesophagus plays a key role in driving metaplastic transformation and may have the potential for halting disease progression through novel therapeutic strategies.

OAC is a detrimental manifestation of inflammation and is thought to be influenced by immune cell infiltration at each stage of disease from oesophagitis to BO and OAC. T cells, B-cells, macrophages and dendritic cells have all been reported in high numbers in BO and OAC [235-237], but their inflammatory profiles, functions and role in the inflammatory process are not yet fully understood. Recent studies have begun to examine the inflammatory profile in GORD and BO [72, 107] and have implicated T cells as initiators of inflammation in the oesophagus [72]. *In vivo* models of reflux disease, have demonstrated that T cells infiltrate the sub-mucosal layers prior to tissue damage and prior to other immune cells, suggesting that they may initiate the inflammatory response and damage in GORD [72]. Furthermore, levels of CCL5, a T cell chemoattractant protein is increased in oesophagitis patients [179], while BO patients exhibit reduced systemic IL-2 production, suggesting impaired T cell activation at this stage of the disease sequence [238]. While there remains a paucity of

studies examining the T cell profile and function in BO, Fitzgerald *et al*, have examined the general cytokine profile and inflammatory cell infiltrate in oesophagitis and BO [107]. They observed increased levels of IL-1 β , IFN- γ and CXCL8 in the tissue of oesophagitis patients compared to controls, whereas BO tissue displayed increased levels of IL-10 and IL-4 exclusively. This suggests that BO is characterised by a Th2 like response while oesophagitis has a more pro-inflammatory cell mediated cytokine profile [107]. An inflammatory shift to a more Th1- response is suggested to occur in OAC with levels of proinflammatory cytokines such as IFN- γ , IL-2, IL-1 β and the chemokine CXCL8 increased compared to healthy controls [105, 106]. While Th1 associated cytokines, like IFN- γ , are known to have anti-angiogenic properties [117], and can stimulate a potent anti-tumor immune response, Th2 like cytokines, such as IL-10 have been shown to stimulate angiogenesis and are therefore considered pro-tumorigenic [123]. Therefore the shift to Th2 during BO may promote the tumourigenesis that manifests as OAC and a better understanding of such underlying immune mechanisms and the crosstalk between the tissue microenvironment and the immune system may facilitate improved prognostic testing and reveal new immunotherapeutic targets. The overall aims of this chapter were as follows:

- To assess the phenotype of T cells across the GORD-BO-OAC sequence; both in circulation and in oesophageal tissue
- To examine the secreted cytokine profile of the oesophageal microenvironment from GORD to BO and OAC
- To examine the impact of secreted factors from the tissue microenvironment on T cell profile and function

3.2 Results

3.2.1 Analysis of T cell activation within oesophageal tissue

A detailed analysis of the T cell activation profile within oesophageal tissue was carried out using *ex-vivo* patient biopsies. Oesophageal biopsies from normal, oesophagitis, BO and OAC tissue were enzymatically digested and stained for CD3, CD4, CD8, CD45RO, CD45RA, CD69 and CD62L. Overall percentages of infiltrating CD4 and CD8 cells did not change across the sequences (Figure 3.1). A significant reduction in the percentage of CD4⁺ T cells expressing CD45RO was observed in tumour tissue compared with normal ($26.2 \pm 10\%$ vs. $69.4 \pm 9.8\%$, $p=0.01$) or Barrett's tissue ($72.2 \pm 3.8\%$, $p=0.007$) (Figure 3.2). Similarly, the percentage of CD4⁺ T cells expressing CD69 was significantly reduced in tumour tissue compared with normal ($9.4 \pm 2.6\%$ vs. $42.6 \pm 9.5\%$, $p=0.002$) (Figure 3.3). A similar result was observed in CD8⁺ T cells although this did not reach statistical significance (Figure 3.3). No alterations were observed in the frequencies of CD45RA⁺ or CD62L⁺ T cells across the disease sequence (Figure 3.4 and Figure 3.5). These results suggest that proportions of activated T cells are decreased within the tumour microenvironment but not at the earlier stages of the disease sequence.

3.2.2 Analysis of T cell cytokine profile within oesophageal tissue

Previous studies have suggested a number of phenotypic switches in the inflammatory response observed throughout the progression from GORD to BO and OAC [11, 15], and have termed these Th1 vs Th2 like responses. However, these studies have not directly assessed the T cell profile within the oesophageal tissue and in order to address this, we examined cytokine production from oesophageal tissue infiltrating T cells by intracellular cytokine staining using flow cytometry. In studies using PMA stimulation, human CD4 cannot be reliably detected, for this study CD4 T cells were identified based on CD3 and CD8 staining (CD3⁺CD8⁻) as this population will consist mainly of CD4⁺ T cells. Henceforth, for studies using PMA and ionomycin staining CD3⁺CD8⁻ cells will be referred to as CD4⁺ T cells. A near significant increase was observed in the percentage of CD4⁺ T cells (CD3⁺CD8⁻) expressing the typical type 1 cytokines, IFN- γ ($21.78 \pm 3.2\%$ vs. $42.7 \pm 11.72\%$, $p=0.06$) (Figure 3.6) however, no alterations were observed in the proportion of cells expressing TNF- α (Figure 3.7). OAC tissue displayed lower IL-12 producing CD4⁺ T cells compared to normal tissue ($2.9 \pm 0.9\%$ vs. $17.3 \pm 6.4\%$, $p=0.1$, NS) however this did not reach significance (Figure 3.8). An

interesting trend was observed with IL-17 producing cells; a significantly reduced proportion of CD4⁺IL-17⁺ cells were observed in oesophagitis ($16.07 \pm 2.4\%$, $p=0.01$) and OAC ($13.07 \pm 5.24\%$, $p=0.03$) tissue but not in BO tissue ($33.68 \pm 10.41\%$) compared to normal ($44.50 \pm 10.18\%$) (Figure 3.9). Interestingly, a significantly higher proportion of IL-4 producing CD4⁺ T cells was observed within BO tissue compared to normal ($16.7 \pm 5.9\%$ vs. $3.6 \pm 1.8\%$, $p=0.03$) and tumour ($2.8 \pm 0.9\%$, $p=0.01$), indicative of a Th2 like response in BO (Figure 3.10). Similarly, higher levels of IL-10 producing CD4⁺ and CD8⁺ T cells were also observed in BO tissue (CD4: $35.1 \pm 11.4\%$; CD8: $28.9 \pm 13.3\%$) compared to normal (CD4: $17.4 \pm 6.9\%$; CD8: $7.9 \pm 2.7\%$, NS), oesophagitis (CD4: $5.9 \pm 2.0\%$; CD8: $5.5 \pm 1.6\%$, $p=0.06$) and OAC (CD4: $11.6 \pm 8.5\%$; CD8: $11.9 \pm 2.0\%$, $p=0.06$), however this did not reach significance (Figure 3.11). This data suggests an increase in Th2 cells within BO tissue which can be considered a pro-tumour phenotype.

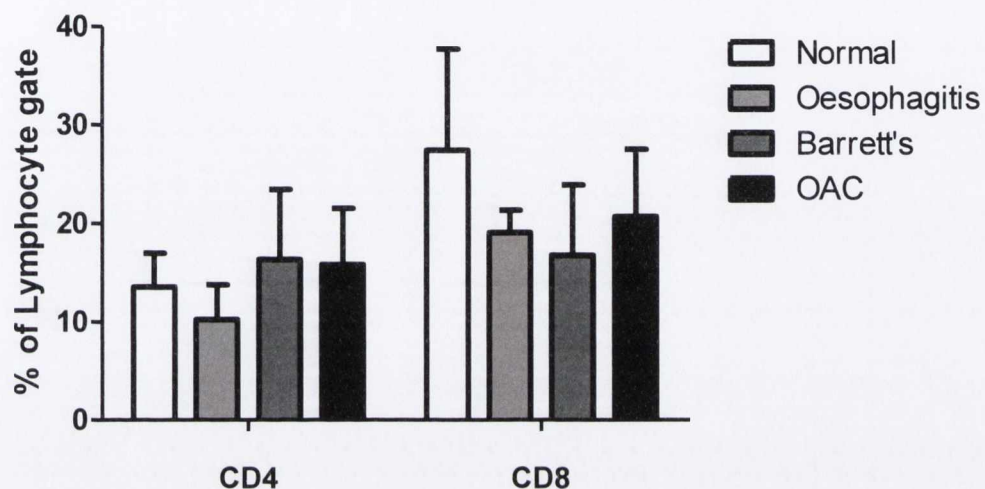


Figure 3.1: Proportion of infiltrating T cell does not change across disease sequence.

Oesophageal biopsies were enzymatically digested and immunophenotyped by flow cytometry. Expression of CD4 and CD8 were measured. Bar chart shows percentage of CD4⁺ and CD8⁺ cells in the lymphocyte gate in Normal, oesophagitis, Barrett's and OAC tissue. All data is presented as mean $\pm$ SEM, n=5-7, One way ANOVA (Kruskal-Wallis), Dunns post-test.

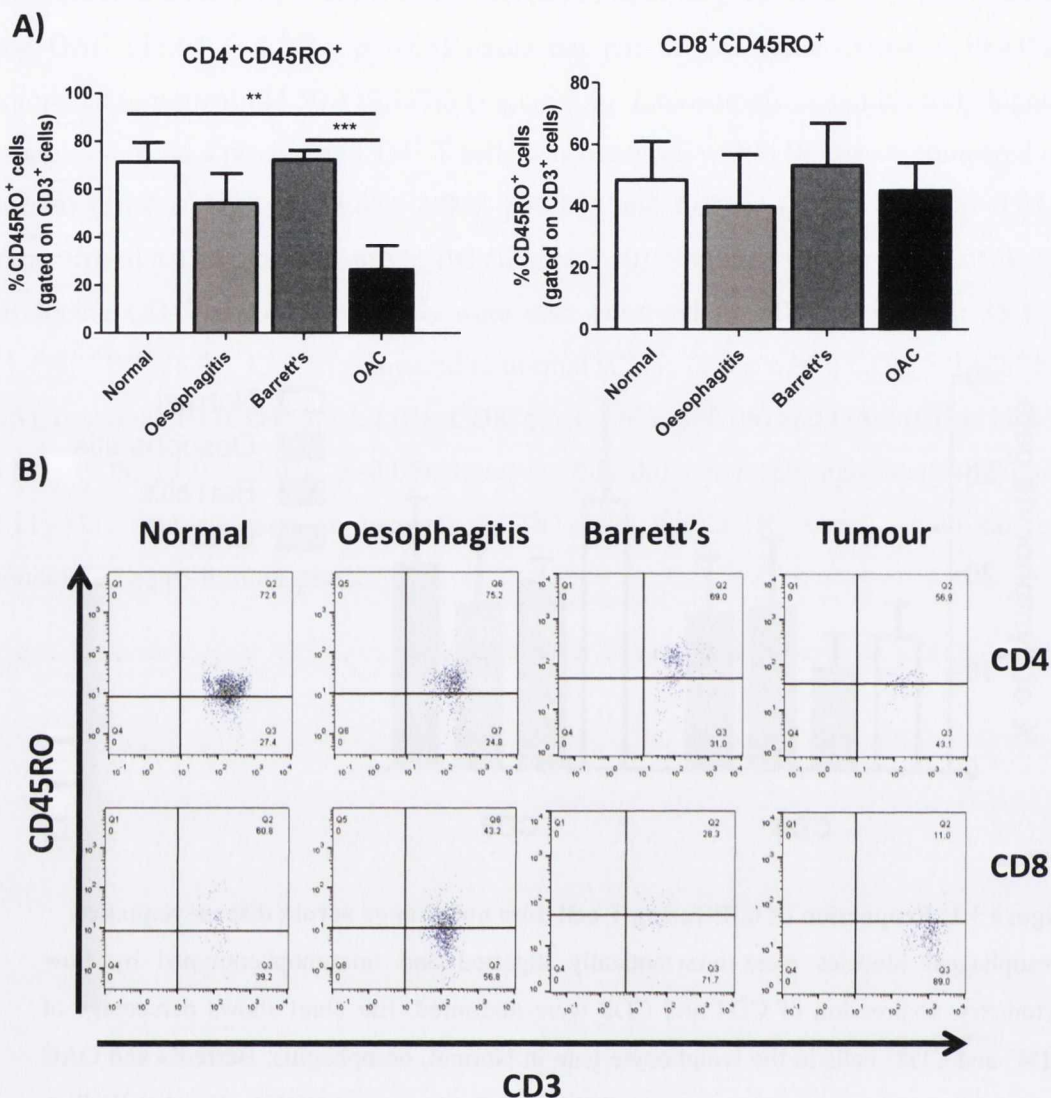


Figure 3.2: Decreased proportion of CD45RO⁺ cells infiltrating OAC tissue.

Oesophageal biopsies were enzymatically digested and immunophenotyped by flow cytometry. Expression of CD4, CD8 and CD45RO were assessed. **A)** Bar charts showing percentage of CD4⁺ or CD8⁺ T cells expressing CD45RO. All data is presented as mean $\pm$ SEM, n=5-7, **p $\leq$ 0.01, ***p $\leq$ 0.001 Students t test (Mann Whitney) **B)** Flow plots show CD45RO expression on CD4⁺ and CD8⁺ cells within normal, oesophagitis, Barrett's and Tumour tissue.

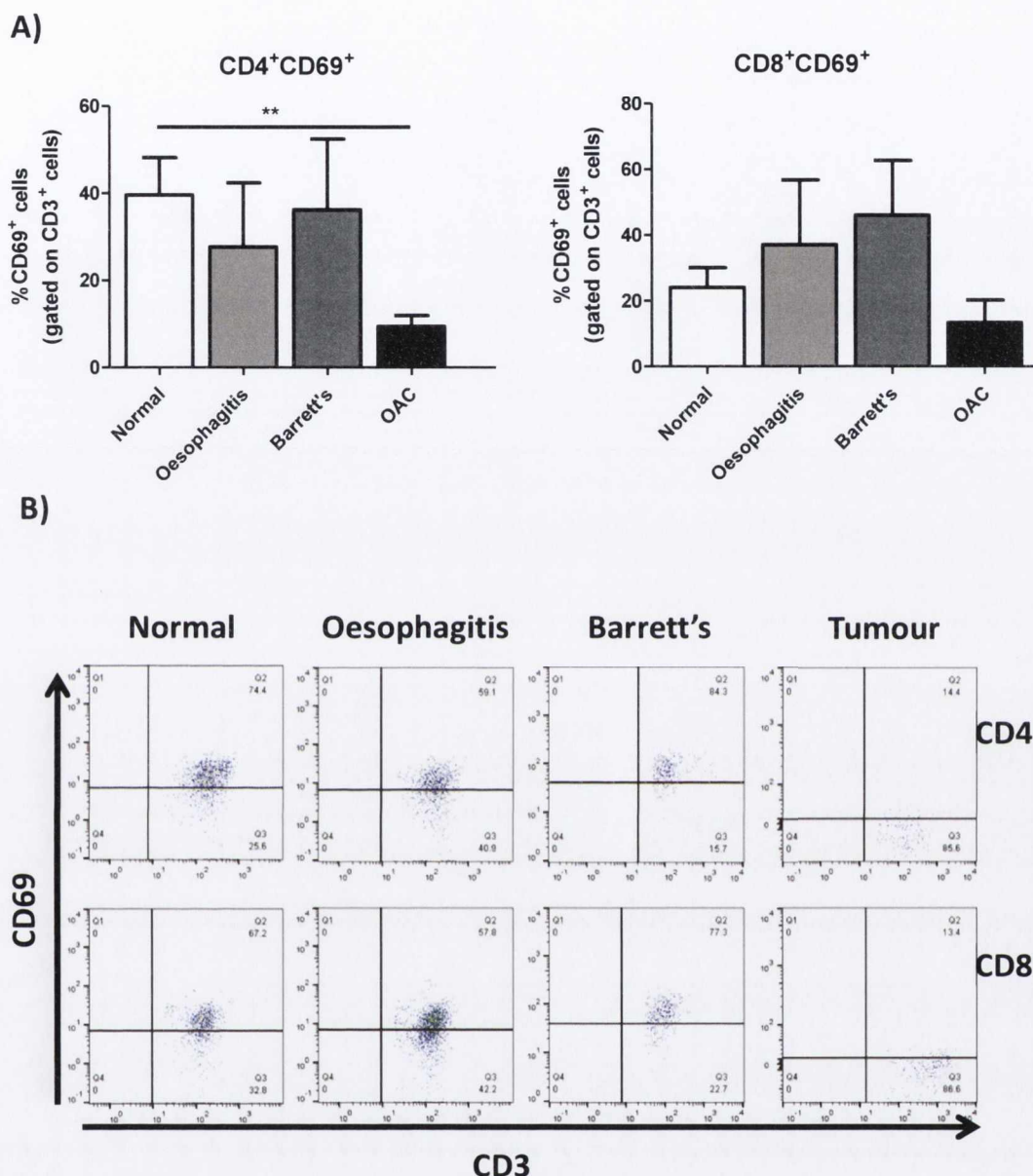
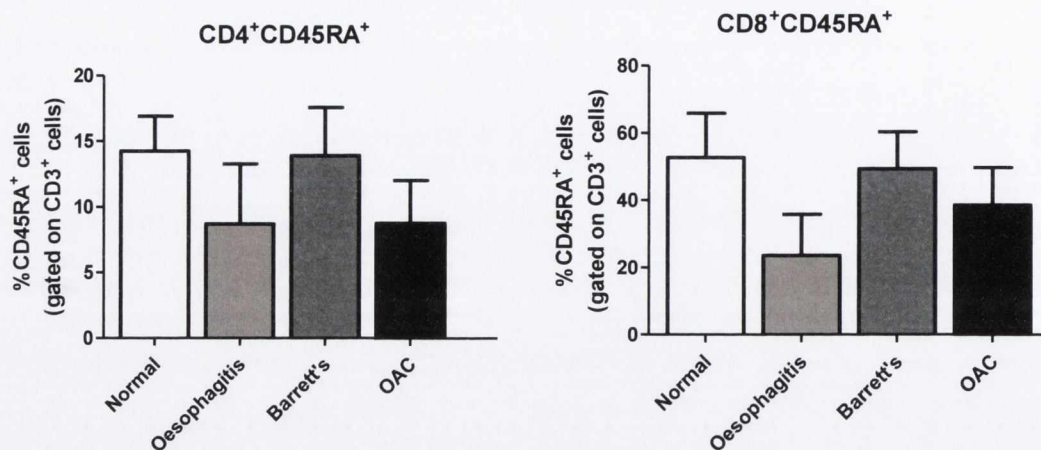


Figure 3.3: Decreased proportion of CD69⁺ T cells infiltrating OAC tissue.

Oesophageal biopsies were enzymatically digested and immunophenotyped by flow cytometry. Expression of CD4, CD8 and CD69 were measured. **A)** Bar charts showing percentage of CD4⁺ or CD8⁺ T cells expressing CD69. All data is presented as mean $\pm$ SEM, n=5-7, **p $\leq$ 0.01, Students t test (Mann Whitney). **B)** Flow plots show CD69 expression on CD4⁺ and CD8⁺ cells within normal, oesophagitis, Barrett's and Tumour tissue.

A)



B)

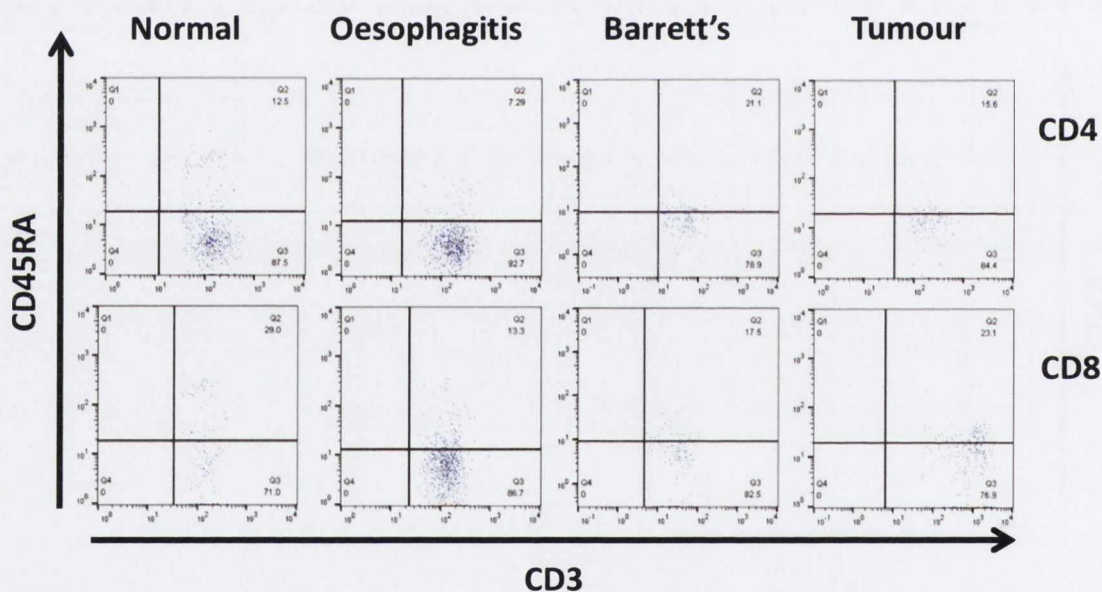


Figure 3.4: CD45RA expression on T cells within oesophageal tissue.

Oesophageal biopsies were enzymatically digested and immunophenotyped by flow cytometry. Expression of CD4, CD8 and CD45RA were measured. **A)** Bar charts showing percentage of CD4⁺ or CD8⁺ T cells expressing CD45RA. All data is presented as mean $\pm$ SEM, n=5-7, Students t test (Mann Whitney). **B)** Flow plots show CD45RA expression on CD4⁺ and CD8⁺ cells within normal, oesophagitis, Barrett's and Tumour tissue.

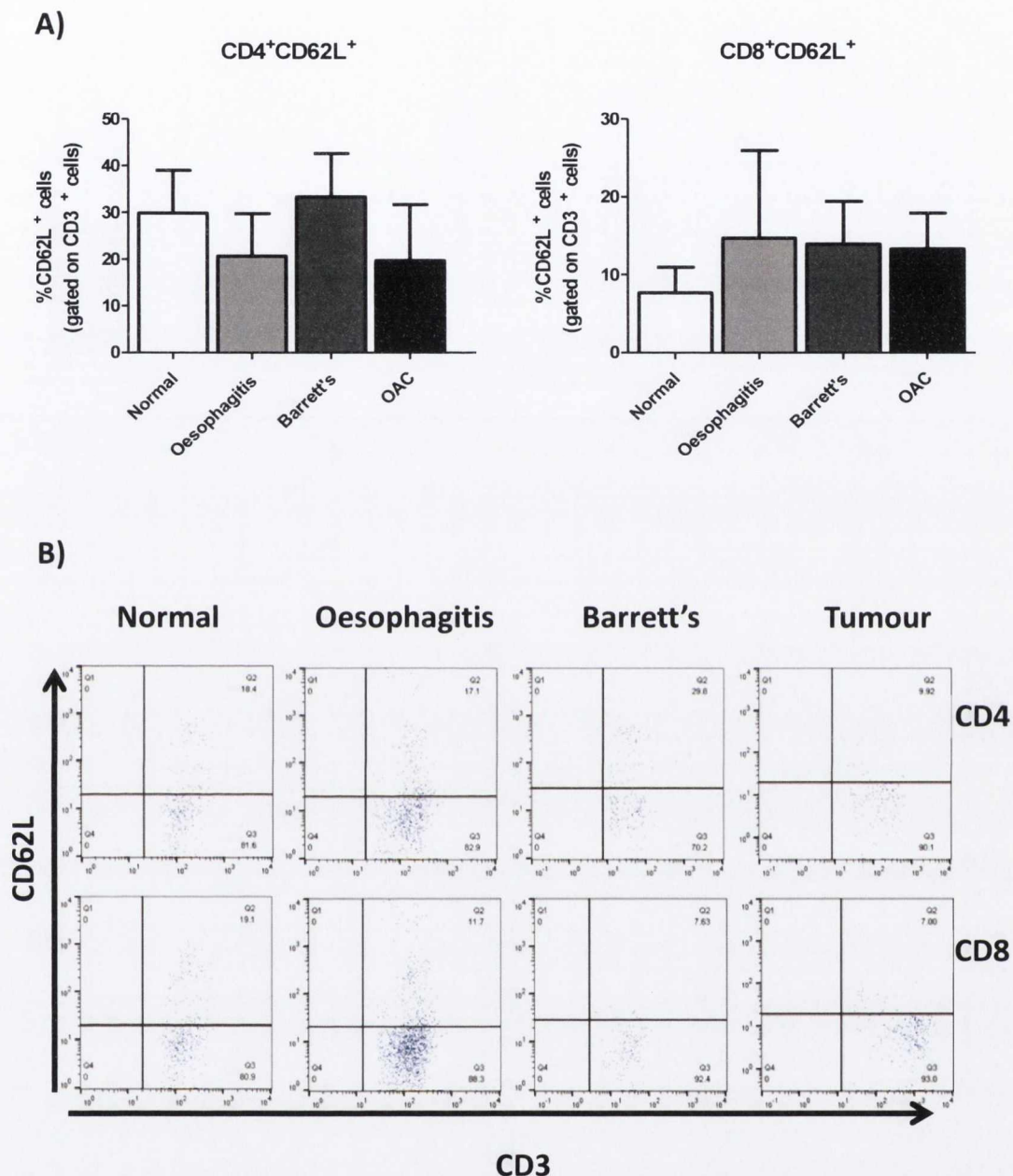


Figure 3.5: CD62L expression on T cells within oesophageal tissue.

Oesophageal biopsies were enzymatically digested and immunophenotyped by flow cytometry. Expression of CD4, CD8 and CD62L were measured. **A)** Bar charts showing percentage of CD4⁺ or CD8⁺ T cells expressing CD62L. All data is presented as mean $\pm$ SEM, n=5-7, Students t test (Mann Whitney). **B)** Flow plots show CD62L expression on CD4⁺ and CD8⁺ cells within normal, oesophagitis, Barrett's and Tumour tissue.

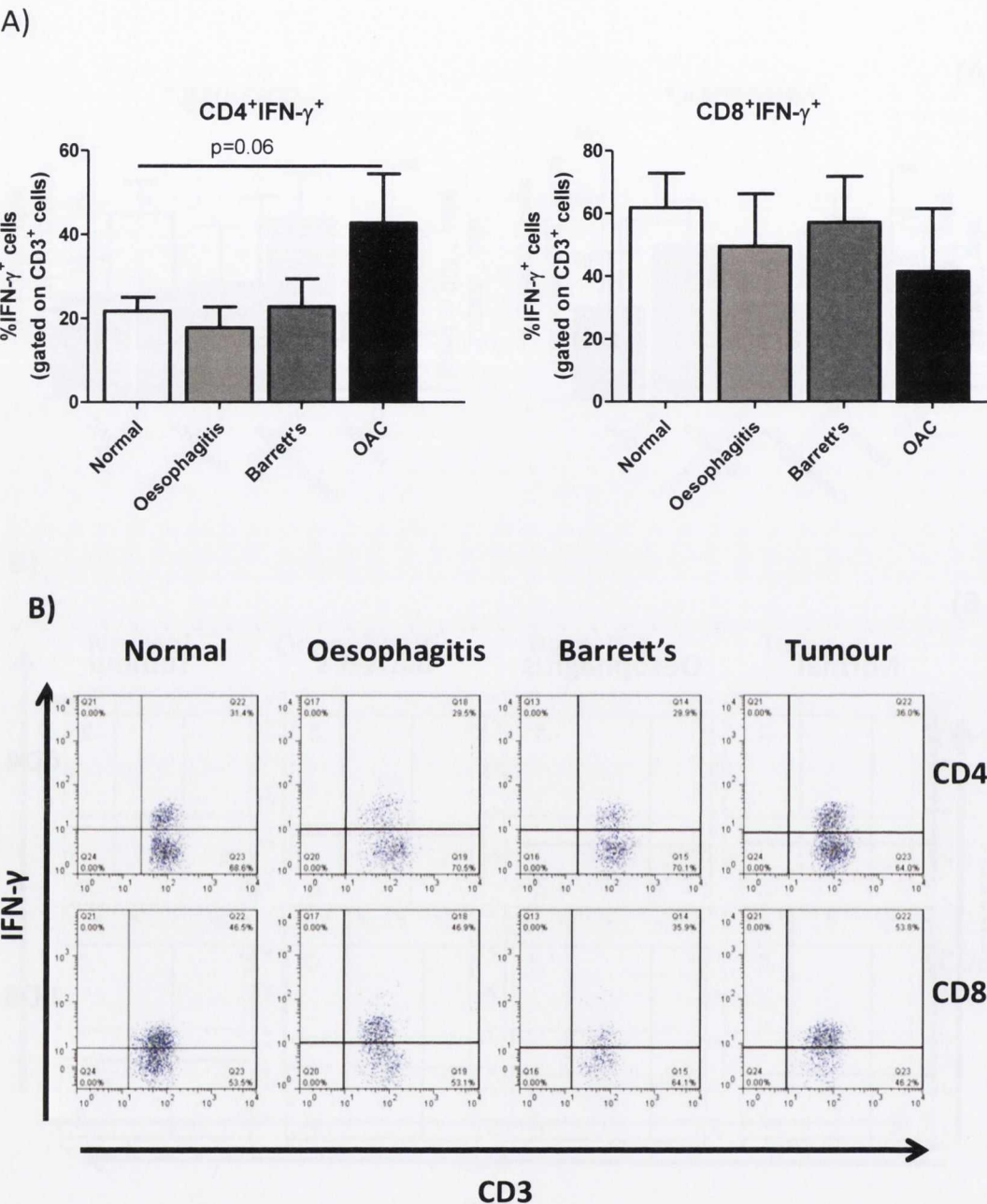


Figure 3.6: IFN- γ expression by oesophageal tissue infiltrating T cells.

Oesophageal biopsies were enzymatically digested and immunophenotyped by flow cytometry. Expression of CD3, CD8 and IFN- γ were measured. A) Bar charts showing percentage of CD4⁺ or CD8⁺ T cells producing IFN- γ . All data is presented as mean $\pm$ SEM, n=4-6, Students t test (Mann Whitney). B) Flow plots show IFN- γ expression on CD4⁺ and CD8⁺ cells within normal, oesophagitis, Barrett's and Tumour tissue.

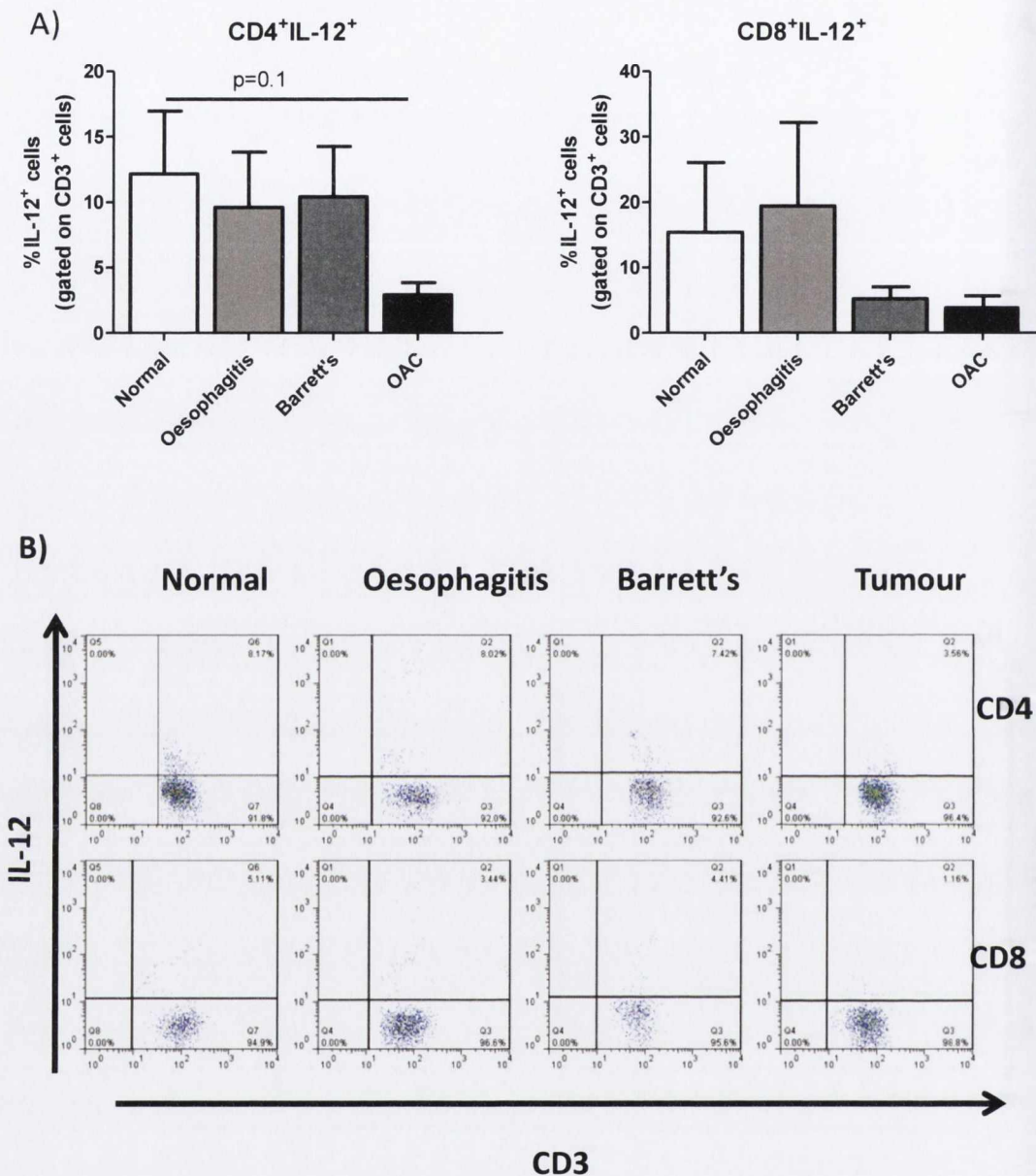
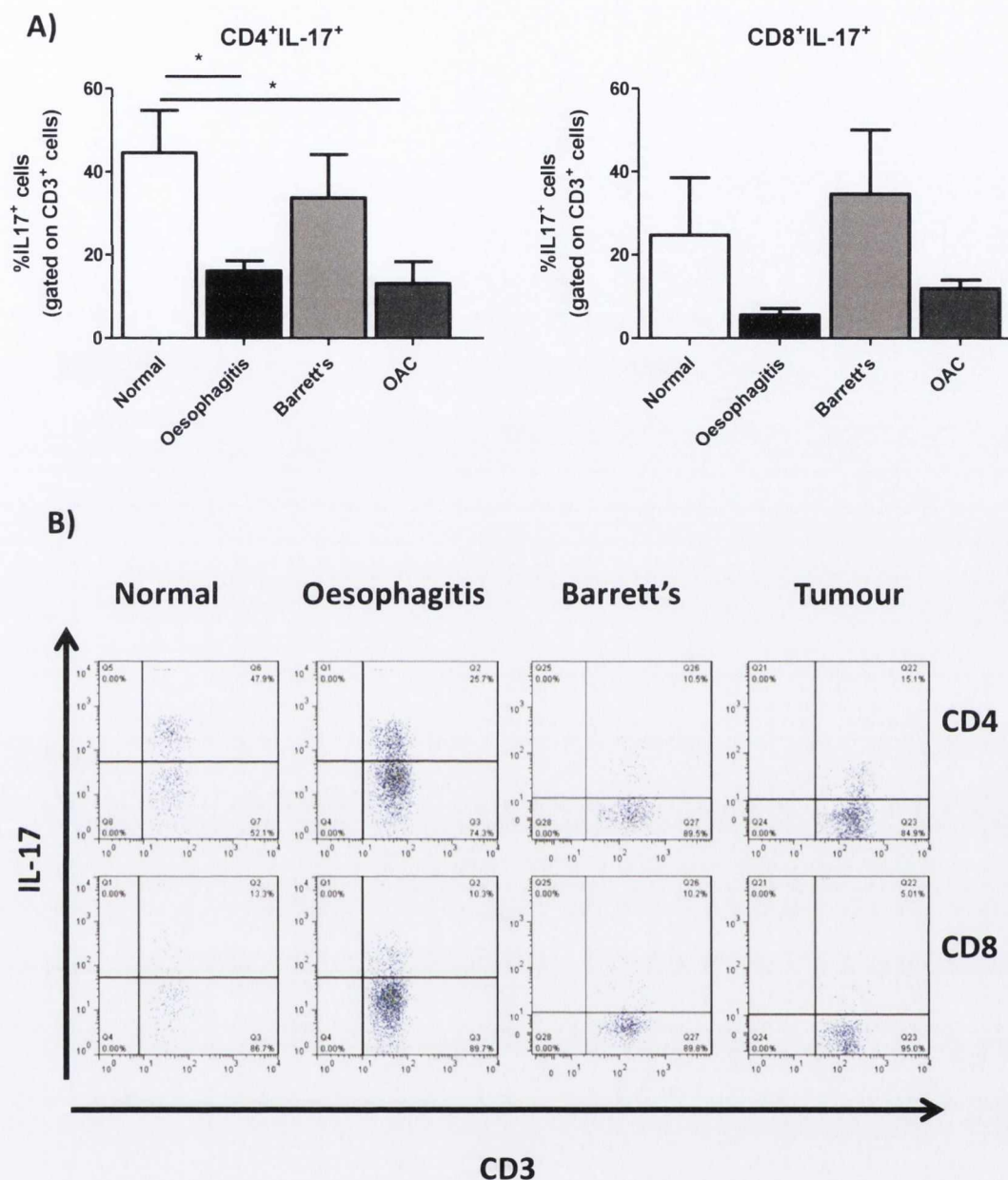


Figure 3.8: IL-12 expression on T cells within oesophageal tissue.

Oesophageal biopsies were enzymatically digested and immunophenotyped by flow cytometry. Expression of CD3, CD8 and IL-12 were measured. A) Bar charts showing percentage of CD4⁺ or CD8⁺ T cells producing IL-12. All data is presented as mean $\pm$ SEM, n=4-6, Students t test (Mann Whitney). B) Flow plots show IL-12 expression on CD4⁺ and CD8⁺ cells within normal, oesophagitis, Barrett's and Tumour tissue.



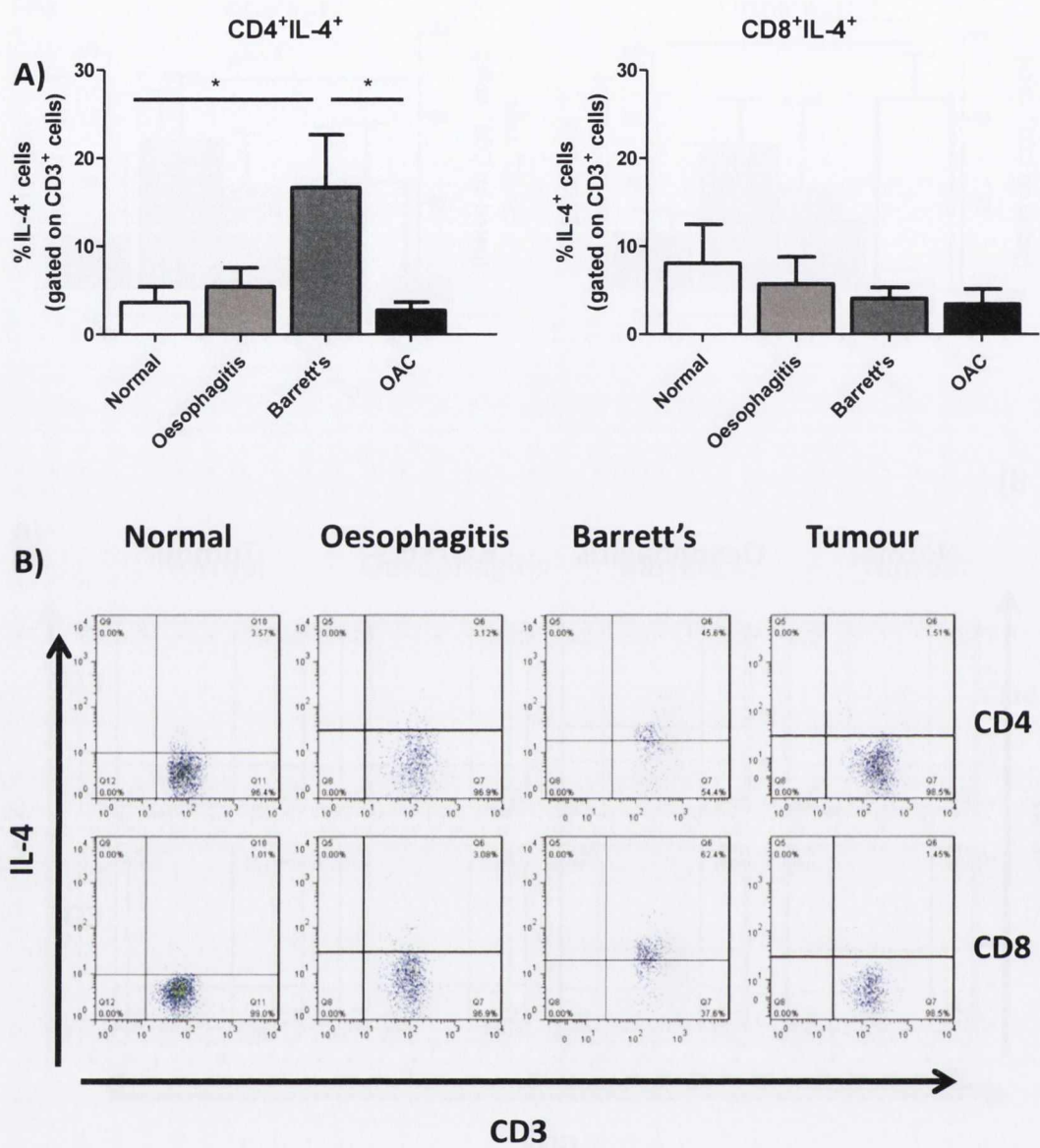
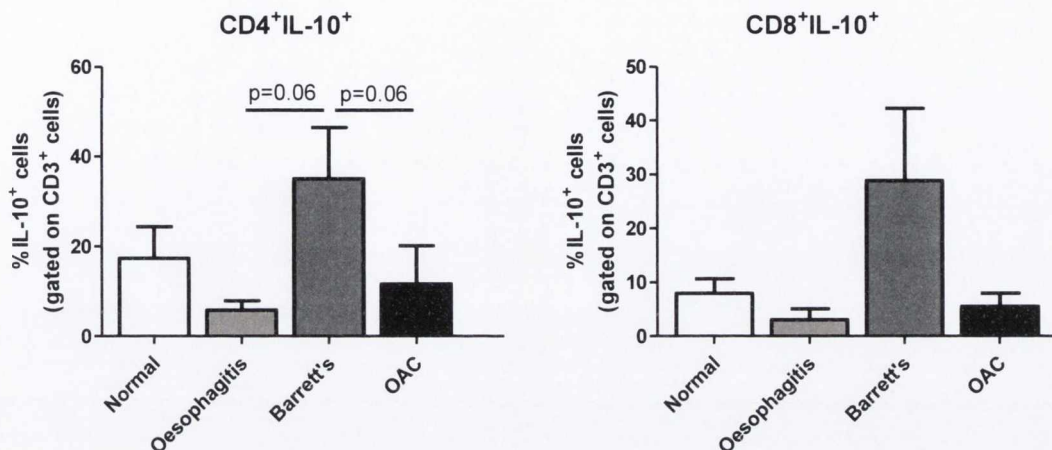


Figure 3.10: Increased IL-4⁺ T cells in BO tissue.

Oesophageal biopsies were enzymatically digested and immunophenotyped by flow cytometry. Expression of CD3, CD8 and IL-4 were measured. A) Bar charts show percentage of CD4⁺ or CD8⁺ T cells producing, IL-4. All data is presented as mean $\pm$ SEM, n=4-6, *p $\leq$ 0.05, Students t test (Mann Whitney). B) Flow plots show IL-4 expression on CD4⁺ and CD8⁺ cells within normal, oesophagitis, Barrett's and Tumour tissue.

A)



B)

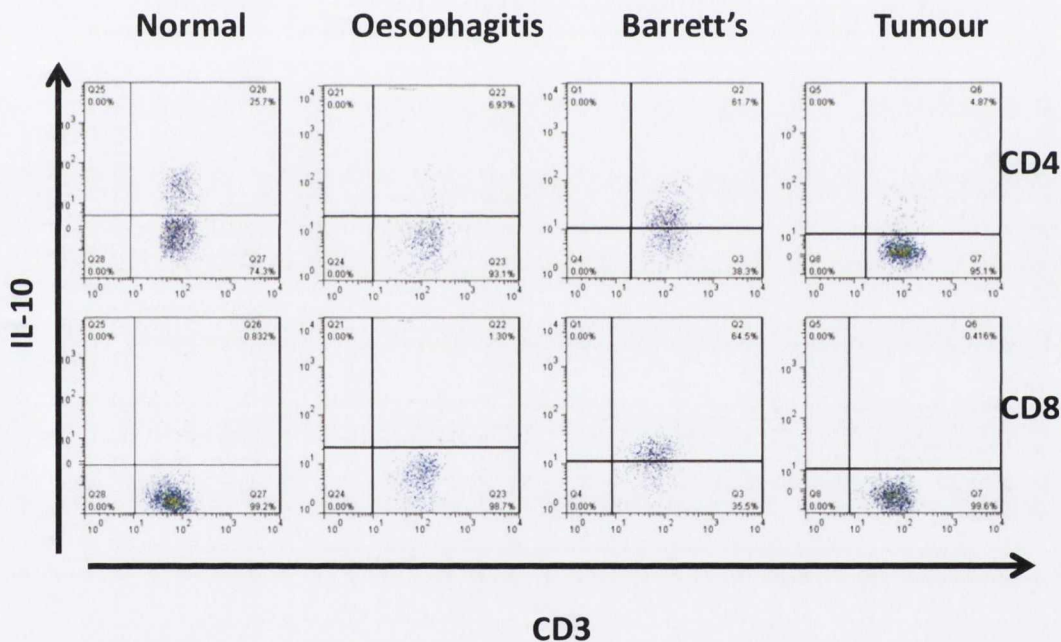


Figure 3.11: IL-10 expression on T cells within oesophageal tissue.

Oesophageal biopsies were enzymatically digested and immunophenotyped by flow cytometry. Expression of CD3, CD8 and IL-10 were measured. A) Bar charts show percentage of CD4⁺ or CD8⁺ T cells producing, IL-10. All data is presented as mean $\pm$ SEM, n=4-6, *p $\leq$ 0.05, Students t test (Mann Whitney). B) Flow plots show IL-10 expression on CD4⁺ and CD8⁺ cells within normal, oesophagitis, Barrett's and Tumour tissue.

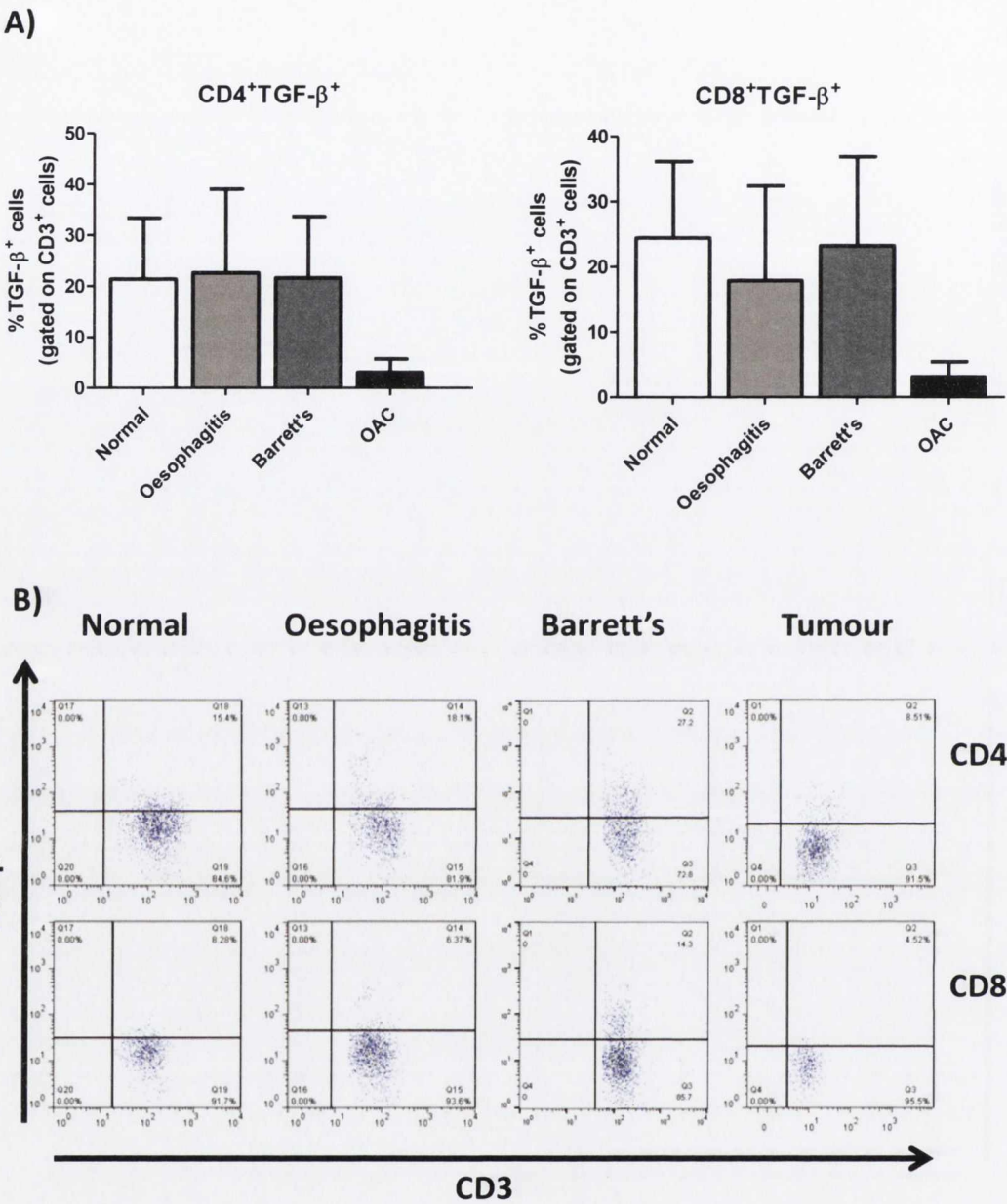


Figure 3.12: TGF-β expression on T cells within oesophageal tissue.

Oesophageal biopsies were enzymatically digested and immunophenotyped by flow cytometry. Expression of CD3, CD8 and TGF-β were measured. A) Bar charts show percentage of CD4⁺ or CD8⁺ T cells producing TGF-β. All data is presented as mean ± SEM, n=4-6, *p≤0.05, Students t test (Mann Whitney). B) Flow plots show TGF-β expression on CD4⁺ and CD8⁺ cells within normal, oesophagitis, Barrett's and Tumour tissue.

3.2.3 Analysis of T cell activation of circulating T cells

A detailed analysis of activation profile of circulating T cells was carried out using blood from normal, oesophagitis, BO and OAC patients. PBMC were isolated and stained for CD3, CD4, CD8, CD45RO, CD45RA, CD69 and CD62L. Significantly lower levels of CD45RA⁺CD8⁺ cells were identified in Barrett's patients (Figure 3.13), suggesting a reduced proportion of naïve T cells at this disease stage. As such levels of CD69⁺ cells were increased in BO patients compared to controls. Significantly higher proportions of CD4⁺CD69⁺ were observed in BO blood ($0.77 \pm 0.12\%$) compared to normal ($0.28 \pm 0.06\%$, $p=0.02$), and OAC ($0.07 \pm 0.03\%$, $p=0.02$). Similarly blood from OAC patients displayed significantly lower proportions of CD8⁺CD69⁺ ($0.11 \pm 0.11\%$) T cells compared to Barrett's patients ($0.95 \pm 0.55\%$, $p=0.02$), suggesting a reduced proportion of activated T cells in OAC patients (Figure 3.13). Levels of CD62L and CD45RO expressing cells were unchanged throughout the sequence. These results suggest that while circulating T cells in BO patients show increased activation; this effect is not maintained in OAC, similar to results observed within OAC tissue.

3.2.4 Analysis of cytokine profile of circulating T cells

To assess intracellular cytokine expression, PBMC were stimulated with PMA/Ionomycin and stained for CD3, CD8 and the intracellular cytokines IFN- γ , TNF- α , IL-12, IL-4 and IL-10. Results demonstrated a significant increase in levels of IL4⁺CD8⁺ T cells in blood from BO patients ($2.69 \pm 0.48\%$) compared to controls ($1.00 \pm 0.26\%$, $p=0.04$), while levels of IL-10⁺CD8⁺ T cells were lower in BO blood ($0.17 \pm 0.03\%$) compared to controls ($0.45 \pm 0.06\%$, $p=0.03$). There was a near significant decrease in the proportion of TNF- α producing T cells in OAC blood compared to controls ($7.95 \pm 4.19\%$ vs $30.95 \pm 6.89\%$, $p=0.06$) (Figure 3.14). No significant differences were observed in IFN- γ or IL-12 producing cells. Results do not demonstrate a clear T cell phenotype in circulating T cells, however the reduced proportion of TNF- α producing cells alongside reduced CD69⁺ cells in OAC blood may represent a systemic reduction in T cell activation and effector function.

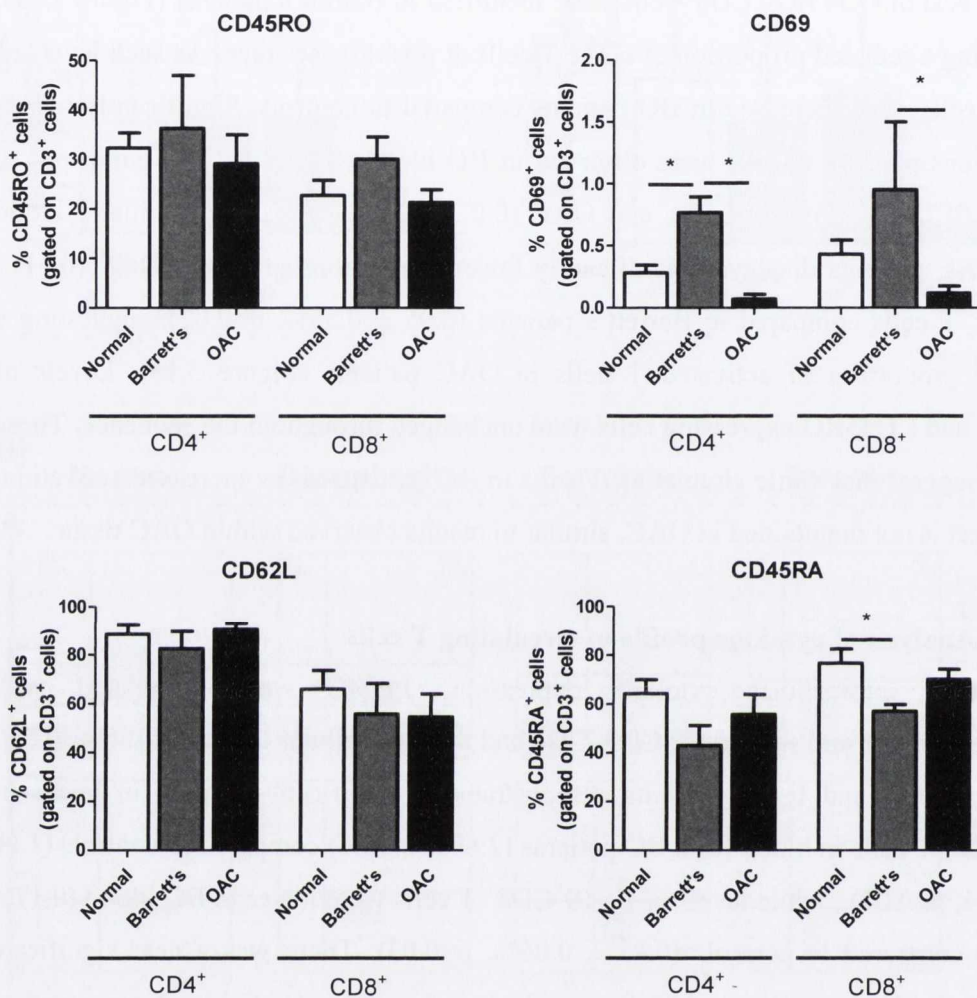


Figure 3.13: Expression of surface activation markers on circulating T cells.

PBMCs were isolated from blood of normal, oesophagitis, Barrett's and OAC patients. Expression of CD3, CD4, CD8 CD45RO, CD45RA, CD69 and CD62L were measured. Bar charts show percentage of CD4⁺ or CD8⁺ T cells producing surface activation markers. All data is presented as mean $\pm$ SEM, n=4, *p $\leq$ 0.05, Students t test (Mann Whitney), *p $\leq$ 0.05.

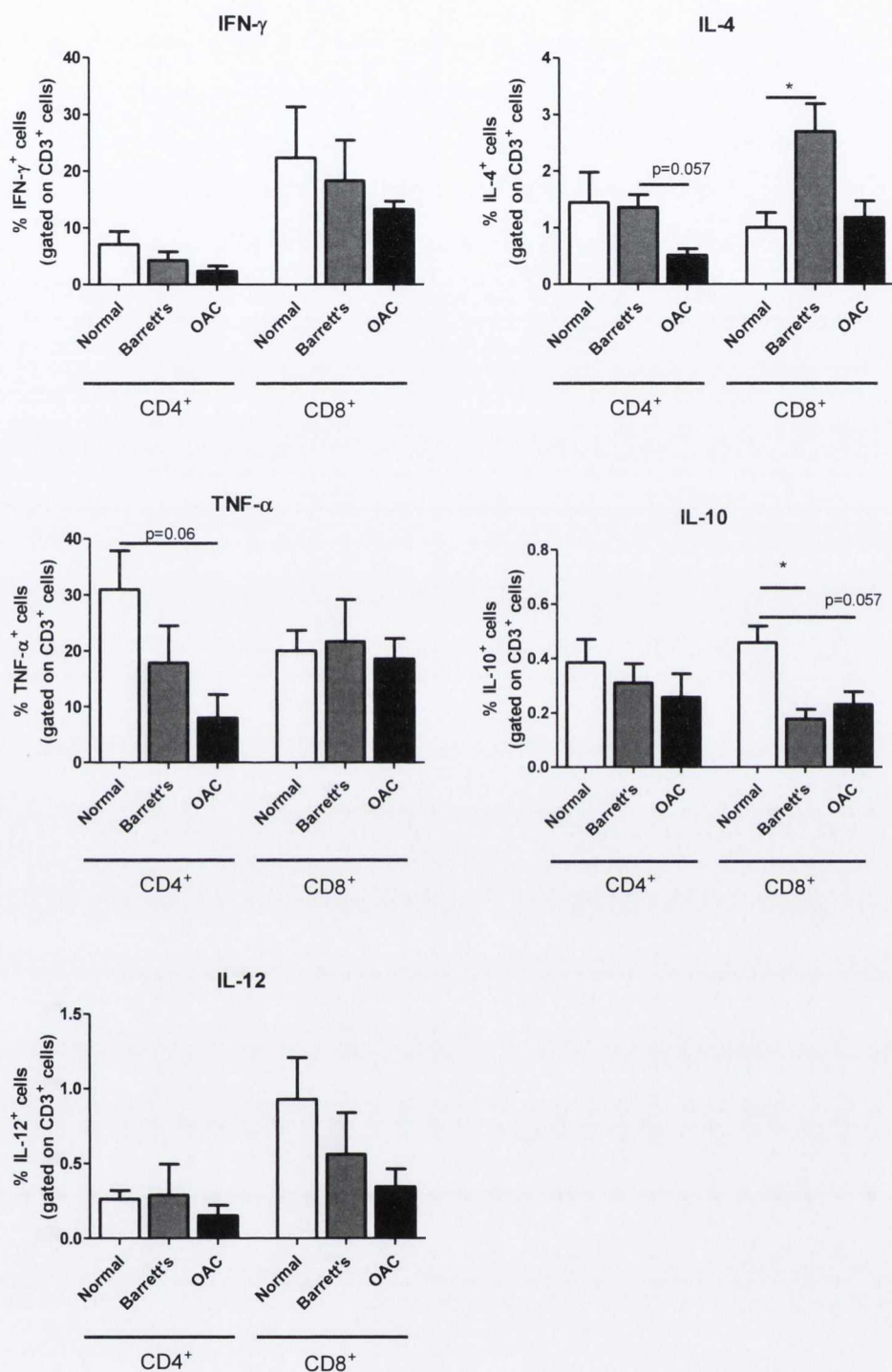


Figure 3.14: Cytokine profile of circulating T cells.

PBMC were isolated from blood of normal, oesophagitis, Barrett's and OAC patients. Expression of CD3, CD8, IFN- γ , TNF- α , IL-12, IL-4 and IL-10 were measured. Bar charts show percentage of CD4⁺ or CD8⁺ T cells producing surface activation markers. All data is presented as mean $\pm$ SEM, n=4, *p $\leq$ 0.05, compared to normal, t-test (Mann Whitney).

3.2.5 Analysis of the secreted cytokines within oesophageal microenvironment

The complex inflammatory microenvironment which exists in oesophagitis, BO and OAC, consists of inflammatory cytokines and immune cells with the potential to alter the phenotype and function of resident T cells. To determine what soluble factors are present in the local microenvironment with known abilities to alter T cell phenotype and function, biopsies were cultured to generate conditioned media (CM); normal (NCM), oesophagitis (OCM), Barrett's (BCM) and tumour (TCM). Since oesophageal biopsies are multicellular, containing epithelial cells, stromal cells and immune cells; conditioned media generated in this manner represents a complex microenvironment, with factors released from various sources. Levels of inflammatory cytokines reported to impact T cell function (IFN- γ , TNF- α , IL-1 β , IL-12p70, IL-2, IL-15, IL-6, IL-10, IL-4, GM-CSF) were measured.

Within the OCM, levels of IFN- γ were higher compared to NCM (4.8 ± 3.1 pg/ μ g vs. 0.24 ± 0.09 pg/ μ g, $p=0.01$). Similar trends were observed with IL-2 (12.9 ± 9.1 pg/ μ g vs. 0.85 ± 0.31 pg/ μ g, $p=0.08$) however, this did not reach significance (Figure 3.15). Within the BO microenvironment levels of IL-1 β (19.82 ± 12.28 pg/ μ g vs. 1.56 ± 0.77 pg/ μ g, $p=0.01$), IL-10 (3.07 ± 1.2 pg/ μ g vs. 0.69 ± 0.41 pg/ μ g, $p=0.01$), IL-6 (1016 ± 233.7 pg/ μ g vs. 175.9 ± 72.9 pg/ μ g, $p=0.002$) and GM-CSF (43.2 ± 24.5 pg/ μ g vs. 4.3 ± 3.1 pg/ μ g, $p=0.001$) were significantly higher compared to NCM (Figure 3.15 and Figure 3.16). The tumour microenvironment contained a vast milieu of cytokines; within the TCM levels of IFN- γ (4.8 ± 1.5 pg/ μ g vs. 0.24 ± 0.09 pg/ μ g, $p=0.0006$), TNF- α (55.8 ± 18.6 pg/ μ g vs. 5.5 ± 1.2 pg/ μ g, $p=0.001$), IL-1 β (121.5 ± 39.2 pg/ μ g vs. 1.56 ± 0.77 pg/ μ g, $p<0.0001$), IL12p70 (2.4 ± 0.93 pg/ μ g vs. 0.45 ± 0.14 pg/ μ g, $p=0.01$), IL-2 (3.5 ± 1.2 pg/ μ g vs. 0.85 ± 0.31 pg/ μ g, $p=0.003$), IL-10 (10.8 ± 3.7 pg/ μ g vs. 0.59 ± 0.41 pg/ μ g, $p=0.0001$), IL-6 (1924 ± 614 pg/ μ g vs. 175.9 ± 72.9 pg/ μ g, $p=0.0002$) and GM-CSF (557.9 ± 394.0 pg/ μ g vs. 4.29 ± 3.1 pg/ μ g, $p<0.0001$) were all significantly higher compared to NCM (Figure 3.15 and Figure 3.16). No differences were observed in IL-4 or IL-15

These results suggest that oesophagitis can be characterised by a pro-inflammatory profile with increased levels of IFN- γ , and BO by a more Th2 like profile with increased IL-6 production. Within the OAC microenvironment the results demonstrate a more complex milieu of cytokines characterised by the expression of Th1/Th2 and anti-inflammatory cytokines.

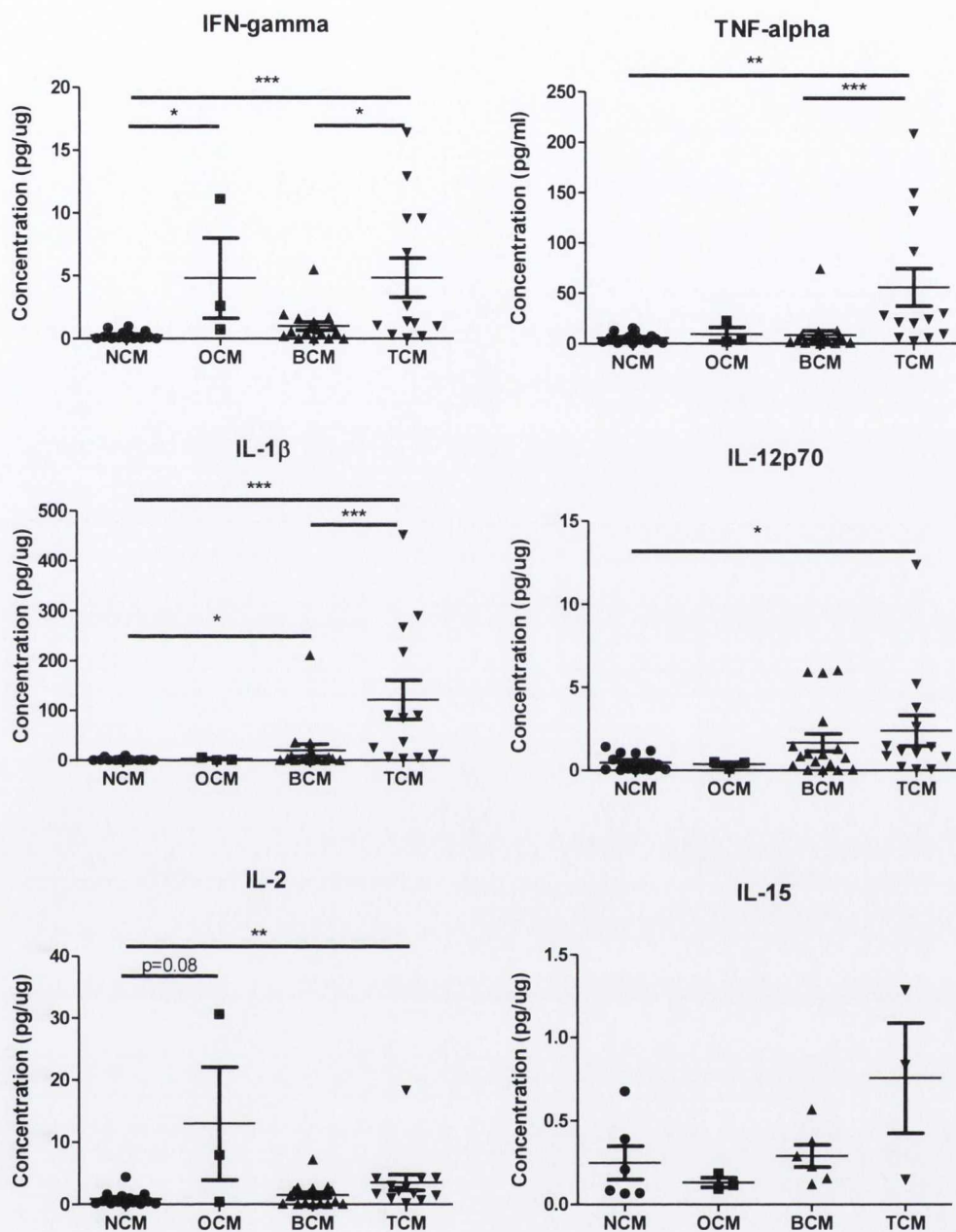


Figure 3.15: Differential cytokine release in tissue microenvironment across disease sequence.

Oesophageal biopsies were cultured in X-vivo media for 24hr to generate oesophageal conditioned media (CM) (NCM=Normal, OCM=Oesophagitis, BCM= Barrett's, TCM= Tumour). Cytokine levels (pg/ml) were measured using MSD multiplex ELISAs and were normalised to total protein in the biopsy ($\mu\text{g/ml}$). All data is presented as mean $\pm$ SEM, * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, t-test (Mann Whitney. $n=14$ NCM, $n=3$ OCM, $n=17$ BCM, $n=13$ TCM).

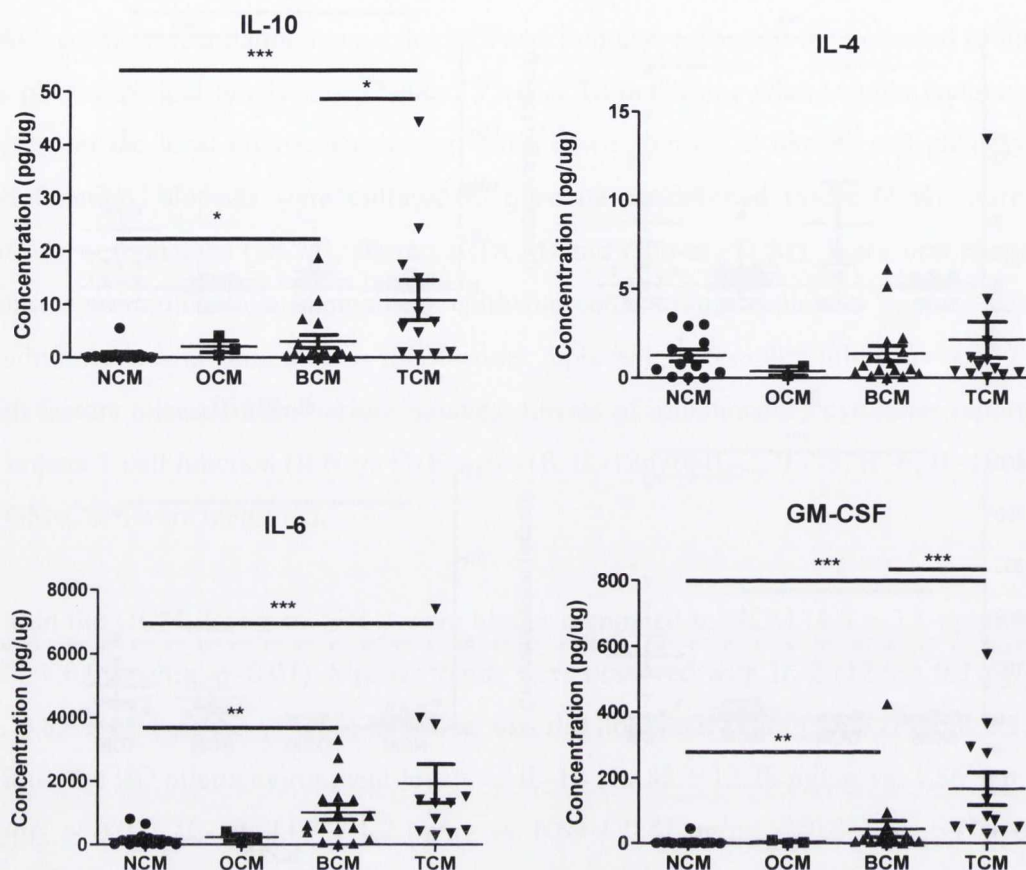


Figure 3.16: Differential cytokine release in tissue microenvironment across disease sequence.

Oesophageal biopsies were cultured in X-vivo media for 24hr to generate oesophageal conditioned media (CM) (NCM=Normal, OCM=Oesophagitis, BCM= Barrett's, TCM= Tumour). Cytokine levels (pg/ml) were measured using MSD multiplex ELISAs and were normalised to total protein in the biopsy ($\mu\text{g/ml}$). All data is presented as mean $\pm$ SEM, * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, t-test (Mann Whitney). $n=14$ NCM, $n=3$ OCM, $n=17$ BCM, $n=13$ TCM.

3.2.6 Analysis of the secreted cytokines from oesophageal epithelial cells

To determine the source of the secreted cytokines, levels were also measured in cell line supernatant (CLS) as a representation of cytokines released solely from an epithelial source, rather than the complex mix in oesophageal biopsies. Interestingly, levels of nearly all cytokines tested (IFN- γ , TNF- α , IL-1 β , IL-12p70, IL-12, IL-15, IL-6, IL-4 and GM-CSF) were found to be higher in QH supernatant (representative of BO) compared to Het-1A (normal) cells (Figure 3.17 and Figure 3.18), most of these remained at the low pg range and may not be therefore biologically relevant. Consistent with the CM however, within the QH supernatant levels of IL-6 were significantly higher ($1106 \pm 470.4\text{pg/ml}$) than in Het-1A ($1.76 \pm 0.185\text{pg/ml}$, $p=0.04$) and OE33 CLS ($1.34 \pm 0.16\text{pg/ml}$, $p=0.04$). Similarly, levels of GM-CSF were found at significantly higher levels in the QH supernatant ($137.3 \pm 9.9\text{pg/ml}$) compared to Het-1A ($0.21 \pm 0.03\text{pg/ml}$, $p=0.0002$) and OE33 CLS ($32.23 \pm 7.46\text{pg/ml}$, $p=0.001$). These results suggest that the predominant source of IL-6 and GM-CSF seen in BCM may come from an epithelial source.

3.2.7 Assessing the effect of secreted factors from oesophageal biopsies on T cell activation and cytokine profile

To determine if secreted factors in the oesophageal microenvironment could alter T cell activation phenotype and cytokine production, PBMC from healthy donors were pre-treated for 24hr with conditioned media (CM) from normal (NCM), oesophagitis (OCM), Barrett's (BCM) and tumour (TCM) biopsies. Flow cytometric analysis revealed that treatment with OCM significantly reduced the proportion of CD45RO⁺CD4⁺ T cells ($41.4 \pm 22.3\%$) compared to NCM ($113.1 \pm 6.3\%$, $p<0.05$) and BCM ($135.5 \pm 19.5\%$, $p<0.01$) (Figure 3.19). Similar results were observed within the CD8⁺ population with OCM treatment reducing CD45RO⁺ cells ($43.8 \pm 20.4\%$) compared to NCM ($114.8 \pm 5.9\%$, $p<0.05$), BCM ($129.7 \pm 15.2\%$, $p<0.01$), and TCM ($104.4 \pm 4.3\%$, $p<0.05$). Following pre-treatment with BCM a significant increase in the proportion of CD69⁺ (CD4: $386.7 \pm 106.5\%$, CD8: $317.5 \pm 84.0\%$) cells compared to OCM was observed (CD4: $72.8 \pm 32.8\%$, $p<0.05$ CD8: $113 \pm 18.8\%$, $p<0.05$) but this effect was lost following treatment with TCM (Figure 3.19). This suggests that T cell function and phenotype are altered very early in oesophageal disease by secreted factors in the local tissue microenvironment, which could pave the way for disease

progression. No significant alterations were observed in the proportion of T cells expressing CD45RA or CD62L following treatment with CM.

A significant reduction in the percentage of IFN- γ CD4⁺ and CD8⁺ cells were observed following treatment with BCM ($73.4 \pm 5.8\%$, $p < 0.05$ and $67.9 \pm 7.6\%$ respectively, $p < 0.05$) compared with media only control) (Figure 3.20), a similar but non-significant reduction was observed following treatment with TCM ($74.3 \pm 3.3\%$, NS and $70.1 \pm 6.2\%$, NS). The percentage of TNF- α producing CD4⁺ and CD8⁺ T cells was also significantly decreased following treatment with BCM (CD4: $63.6 \pm 5.4\%$, $p < 0.01$ and CD8: $68.8 \pm 7.6\%$, $p < 0.05$) and TCM (CD4: $69.1 \pm 4.2\%$, $p < 0.01$ and CD8: $76.2 \pm 4.6\%$, $p < 0.05$). This effect was independent of cell death as measured by PI staining. There was no significant change in PI⁺ cells following treatment with conditioned media. This reduction in Th1 cells again reflects the predominance of the Th2 like profile within Barrett's tissue.

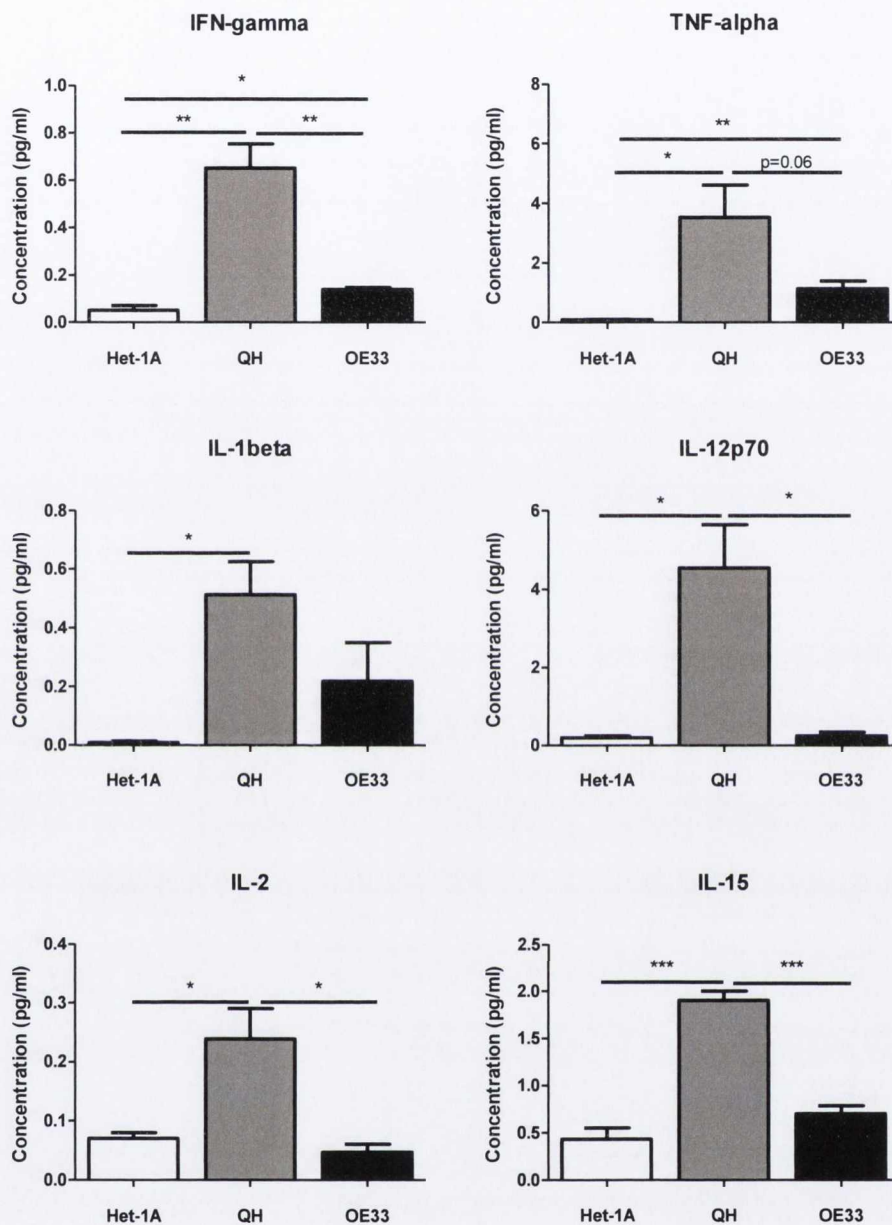


Figure 3.17: Differential cytokine release from oesophageal cell lines.

Cell lines were grown for 4 days until 80% confluent, supernatant was removed and cytokine levels were measured using MSD multiplex ELISAs. All data is presented as mean $\pm$ SEM, * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, unpaired t-test compared, $n=3-6$.

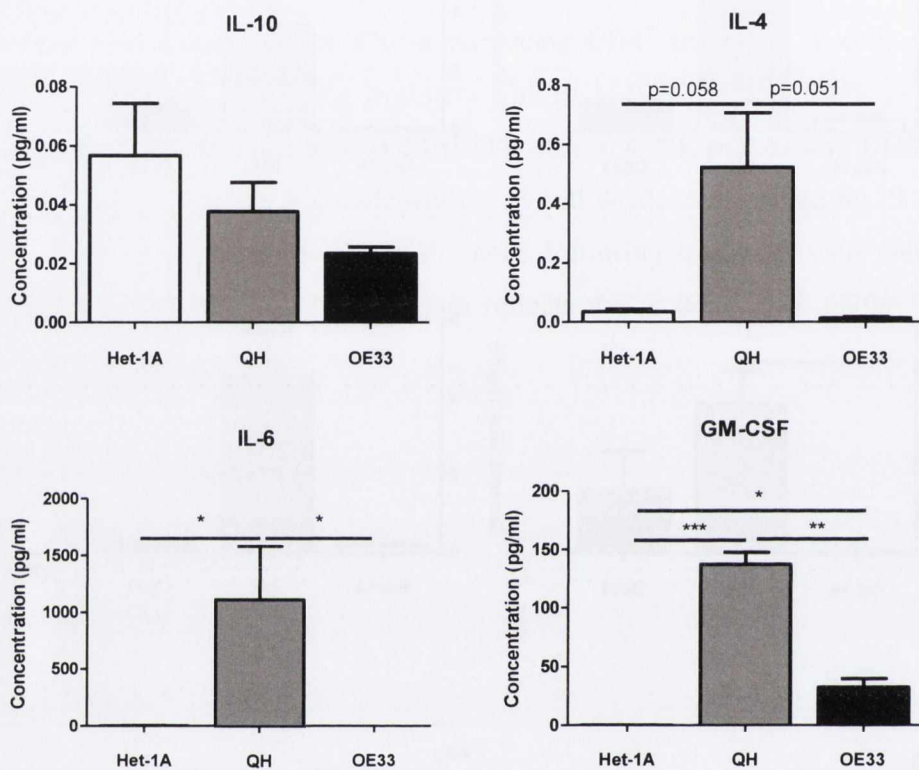


Figure 3.18: Differential cytokine release from oesophageal cell lines.

Cell lines were grown for 4 days until 80% confluent, supernatant was removed and cytokine levels were measured using MSD multiplex ELISAs. All data is presented as mean $\pm$ SEM, * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, unpaired t-test, $n=3-6$.

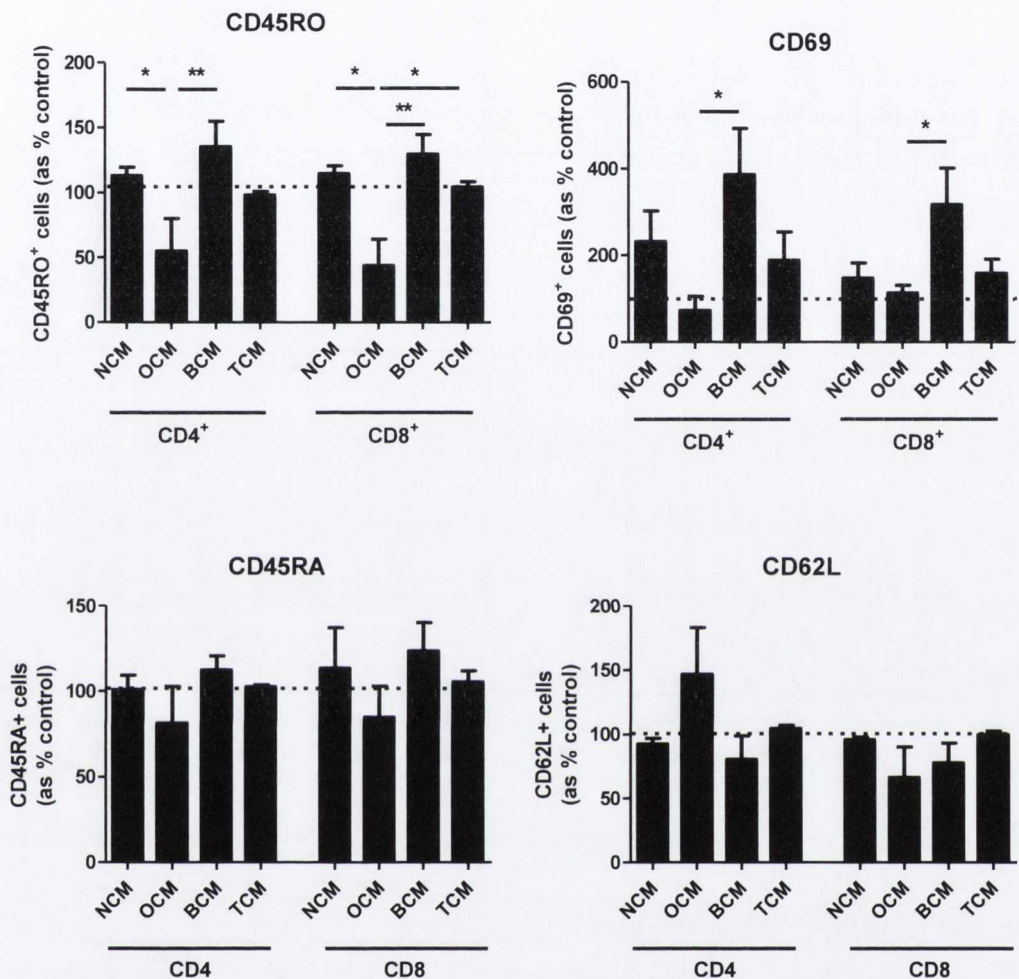


Figure 3.19: Effect of local microenvironment on T cell activation.

PBMCs were isolated from blood and were pre-treated with tissue conditioned media for 24hrs and immunophenotyped by flow cytometry. Bar charts show percentage of CD4⁺ and CD8⁺ T cells expressing CD45RO, CD69, CD45RA and CD62L relative to media only control (dotted line). All data is normalized to media only control (dotted line) and is presented as mean $\pm$ SEM, * $p \leq 0.05$ between groups, One-way ANOVA, Dunn post-test $n=3-4$. Paired t test compared to media only control.

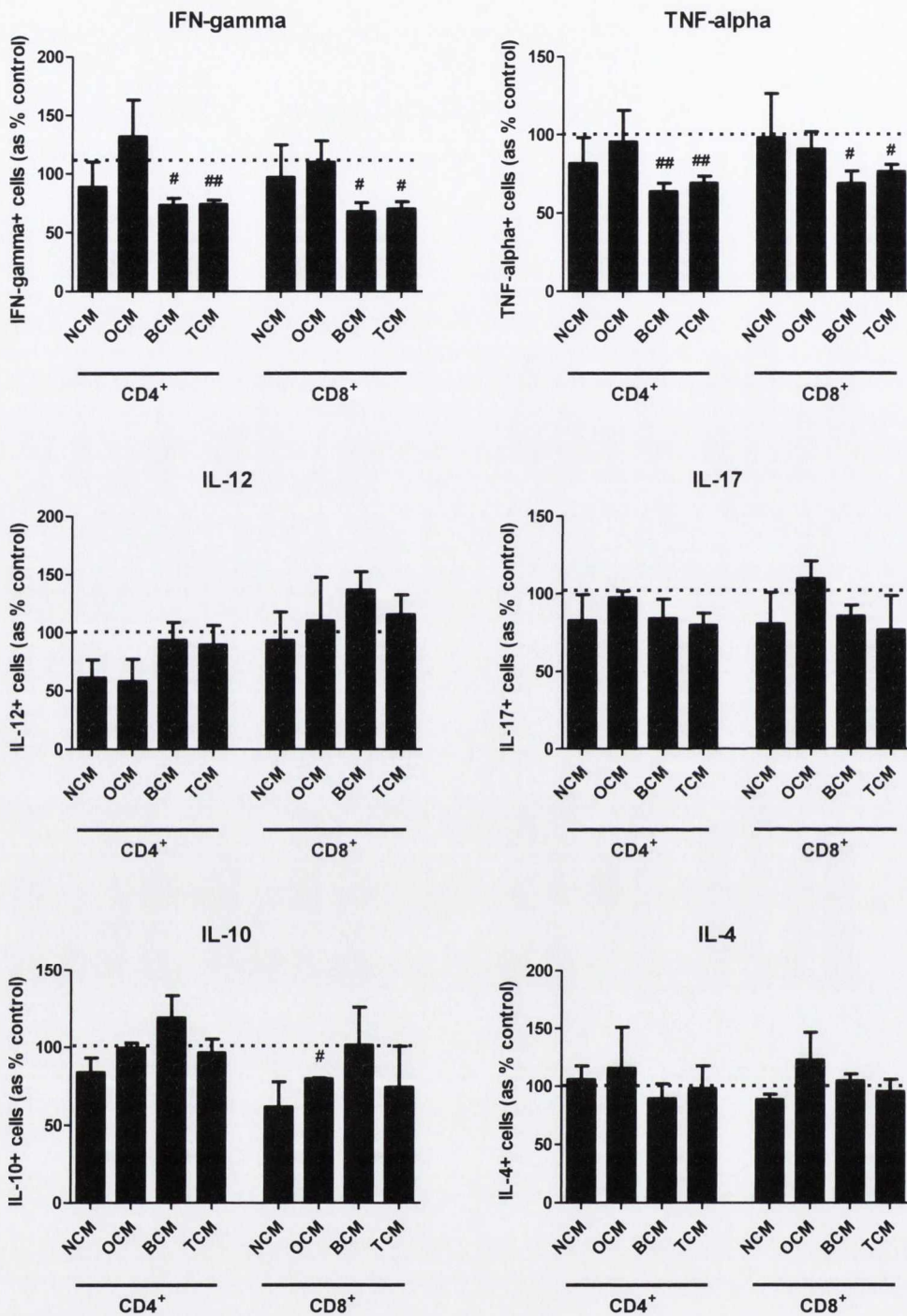


Figure 3.20: Effect of local microenvironment on IC cytokine expression.

PBMCs were isolated from blood and were pre-treated with tissue conditioned media for 24hrs and immunophenotyped by flow cytometry. A) Representative dot plots showing gating strategy used. Bar charts show percentage of CD4⁺ and CD8⁺ T cells expressing IFN- γ , TNF- α , IL-12, IL-10, IL-4 and IL-17 relative to media only control (dotted line). All data is normalized to media only control (dotted line) and is presented as mean $\pm$ SEM, #p $\leq$ 0.05 compared to media only control, paired t-test, n=3-4.

3.2.8 Assessing the effect of epithelial derived secreted factors on T cell activation and cytokine profile

To assess the impact of epithelial derived secreted factors on T cell activation profile and cytokine production, healthy PBMC were pre-treated with supernatant from 3 cell lines: Het-1A, QH and OE33. Similar to results from esophageal biopsy CM, flow cytometric analysis revealed that pre-treatment with QH cell line (Barrett's) supernatant led to an increase in $CD69^+CD4^+$ T cells ($183.1 \pm 33.2\%$, $p=0.08$) compared to media only control, however this effect was lost and a significant reduction was seen following treatment with OE33 CLS ($89.59 \pm 22.71\%$). Within the $CD8^+$ population, OE33 CLS was shown to cause a significant increase in CD69 expression ($158.1 \pm 15.59\%$) compared to media only control (Figure 3.21). Treatment with OE33 CLS caused a significant reduction in levels of CD45RO expressing $CD4^+$ T cells ($90.03 \pm 5.69\%$) when compared with QH CLS ($106.1 \pm 2.57\%$) treated cells (Figure 3.21). No significant alterations were seen in CD62L or CD45RA expression.

A significant reduction in the proportion of $TNF-\alpha$ producing $CD4^+$ cells was seen following pre-treatment with QH ($27.54 \pm 11.44\%$) and OE33 CLS ($37.56 \pm 3.55\%$) compared to media only control (100%). Similar reductions $CD8^+$ T cells were also seen following treatment with QH CLS ($42.66 \pm 24.02\%$, ns). Results from $IFN-\gamma$ and IL-12 producing cells followed a similar trend. The levels of $IFN-\gamma$ producing $CD4^+$ T cells decreased significantly upon treatment with QH CLS ($39.01 \pm 23.53\%$) compared to media only control (Figure 3.22). IL-12 producing $CD4^+$ and $CD8^+$ T cells were also found to significantly decrease following treatment with QH ($74.07 \pm 30.97\%$ and $45.27 \pm 6.5\%$ of control respectively) and OE33 supernatant ($28.19 \pm 2.62\%$ and $74.35 \pm 13.78\%$, ns respectively) (Figure 3.22).

Similarly, levels of IL-10 producing $CD4^+$ and $CD8^+$ T cells decreased following treatment with QH ($49.45 \pm 15.43\%$ and $35.01 \pm 21.71\%$ of control respectively) and OE33 ($28.33 \pm 9.21\%$ and $42.28 \pm 1.97\%$ of control) cell line supernatant (Figure 3.22). A significant reduction in $CD4^+$ and $CD8^+$ T cells expressing IL-4 was demonstrated following treatment with QH (27.09 ± 1.98 and 17.24 ± 3.43) and OE33 cell line supernatant (23.96 ± 5.56 and 28.51 ± 15.63) compared to media only control (Figure 3.22). As expected ssupernatant from the Het-1A cells (normal epithelial cells), caused no significant change in any markers. These results suggest that secreted factors

released by epithelial cells across the disease sequence can alter cytokine production by T cells.

3.2.9 Determining the effect of secreted factors on T cell proliferation

It is possible that alterations in T cell phenotype in GORD, BO and OAC are due to the expansion of resident T cells. To determine whether soluble factors secreted from oesophageal biopsies could alter T cell proliferation, PBMC were cultured with CM for 5 days. Treatment with NCM elicited no effect on T cell proliferation, while OCM caused a significant increase in the percentage of dividing CD8⁺ cells (0.5 ± 0.05 fold increase, $p < 0.05$) and a consistent but not significant increase in CD4⁺ T cell proliferation (0.5 ± 0.2 fold increase, NS). Following treatment with BCM and TCM, there was a progressive decrease in proliferation, similar to levels observed with NCM treatment (Figure 3.23 and Figure 3.24). This suggests secreted factors from the earlier stages of disease have the ability to profoundly affect T cell responses through alterations in their proliferative capacity. In oesophagitis characteristic T cell profiles may be due to the expansion of resident T cells however, in BO results suggest that other factors may be at play.

Interestingly pre-treatment with CLS showed different results. Treatment of both CD4⁺ and CD8⁺ T cells with Het-1A supernatant caused a significant reduction in proliferation (0.37 ± 0.11 fold reduction; 0.26 ± 0.096 fold reduction), compared to media only controls and this reduction was not observed following treatment with QH supernatant (Figure 3.25 and Figure 3.26). This suggests that factors released from the normal (het-1A) epithelial cells may play a homeostatic role preventing T cell proliferation, however the same may not be seen *in-vivo*. A small but significant increase in proliferation was observed with OE33 supernatant (0.027 ± 0.018 fold increase; 0.061 ± 0.02).

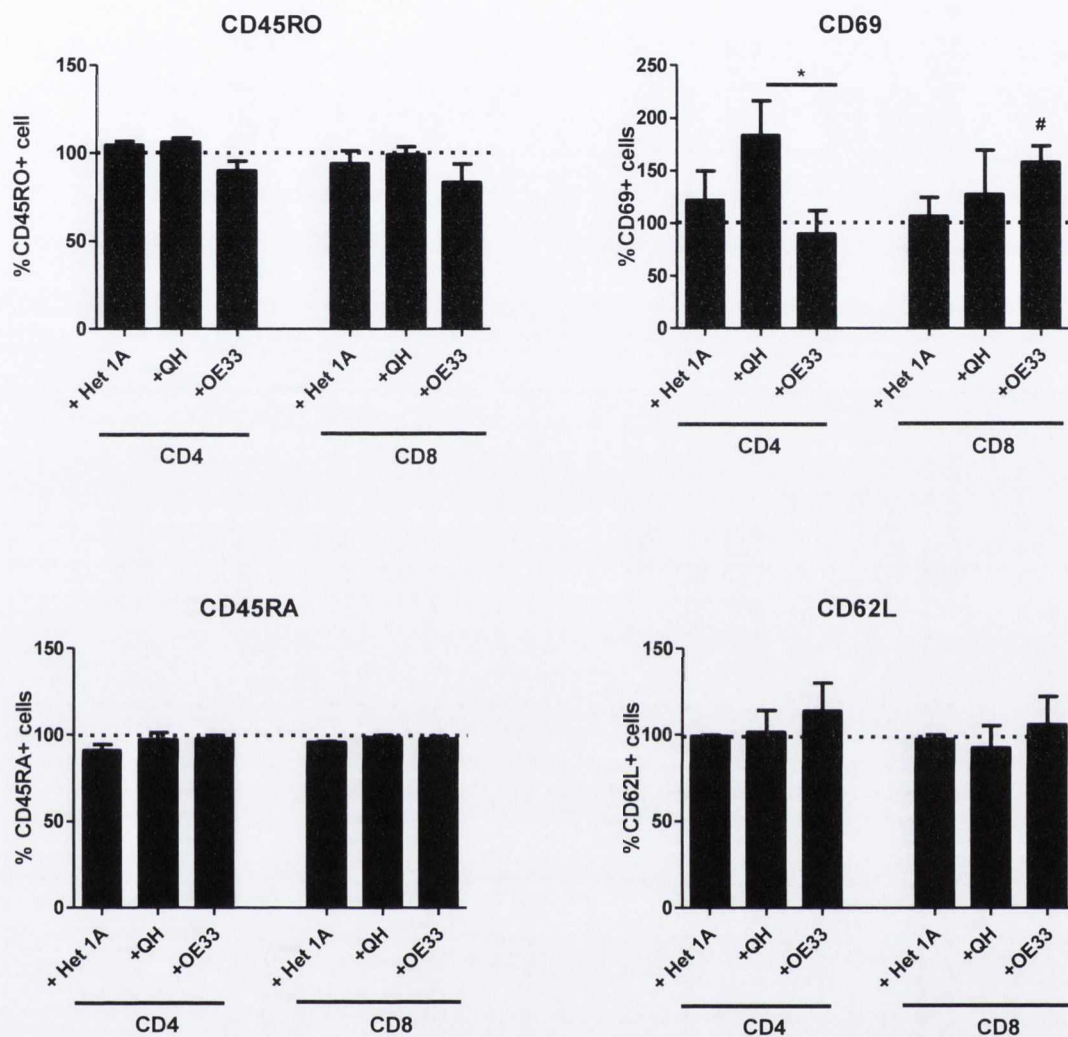


Figure 3.21: Effect of Cell line supernatant on T cell activation.

PBMCs were isolated from blood and were pre-treated with cell line supernatant for 24hrs and immunophenotyped by flow cytometry. Bar charts show percentage of CD4+ and CD8+ T cells expressing CD45RO, CD69, CD45RA and CD62L relative to media only control (dotted line). All data is normalized to media only control (dotted line) and is presented as mean $\pm$ SEM, * $p \leq 0.05$ between groups, One-way ANOVA, Dunn post-test, # $p \leq 0.05$ compared to media only control, paired t-test $n=4-5$.

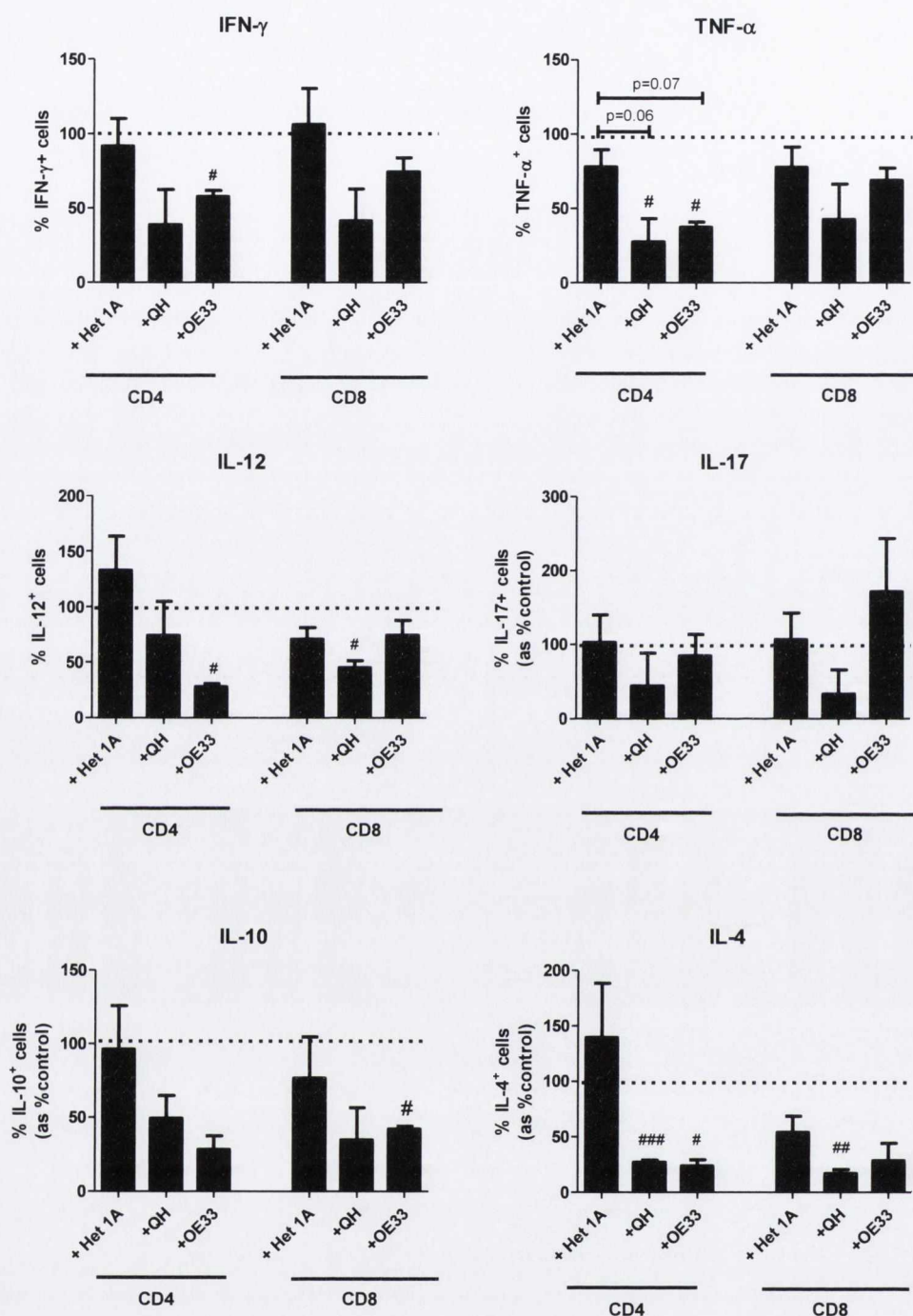


Figure 3.22: Effect of Cell line supernatant on IC cytokine expression.

PBMCs were isolated from blood and were pre-treated with cell line supernatant for 24hrs and immunophenotyped by flow cytometry. A) Representative dot plots showing gating strategy used. Bar charts show percentage of CD4+ and CD8+ T cells expressing IFN- γ , TNF- α , IL-12, IL-10, IL-4 and IL-17 relative to media only control (dotted line). All data is normalized to media only control (dotted line) and is presented as mean $\pm$ SEM, * $p \leq 0.05$ between groups, One-way ANOVA, Dunn post-test, # $p \leq 0.05$ compared to media only control, paired t-test $n=4-5$.

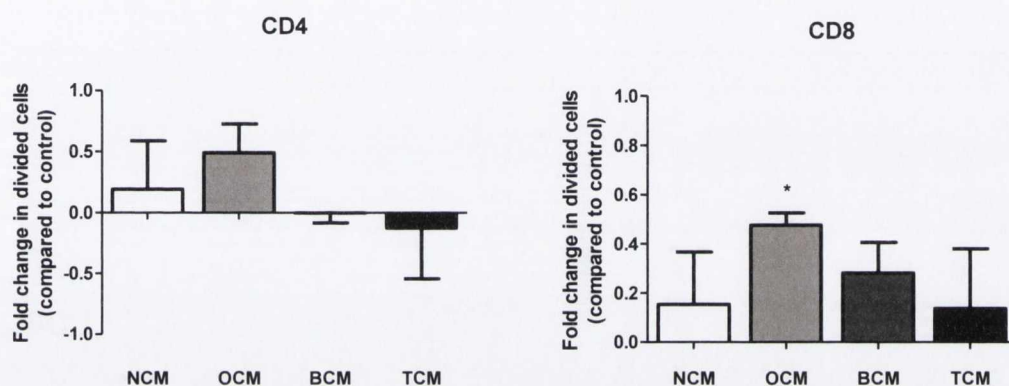


Figure 3.23: Effect of tissue microenvironment on T cell proliferation.

PBMCs were isolated from blood and stimulated with PMA and ionomycin in the presence of tissue conditioned media. Control cells were treated with media only. Proliferation was measured by CFSE staining after 5 days in culture. A) Representative dot plots showing gating strategy. B) Bar charts showing % dividing cells. All data is expressed as fold change in % cells divided compared to media only control $\pm$ SEM. * $p \leq 0.05$, compared to media only control, paired t test, $n=4$.

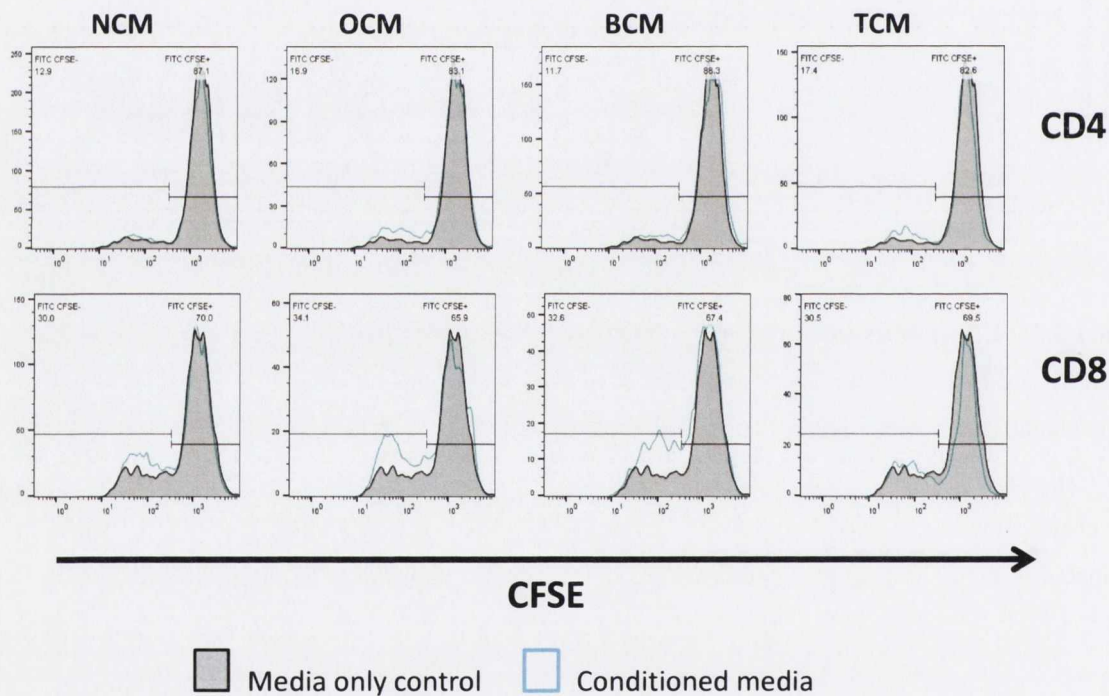
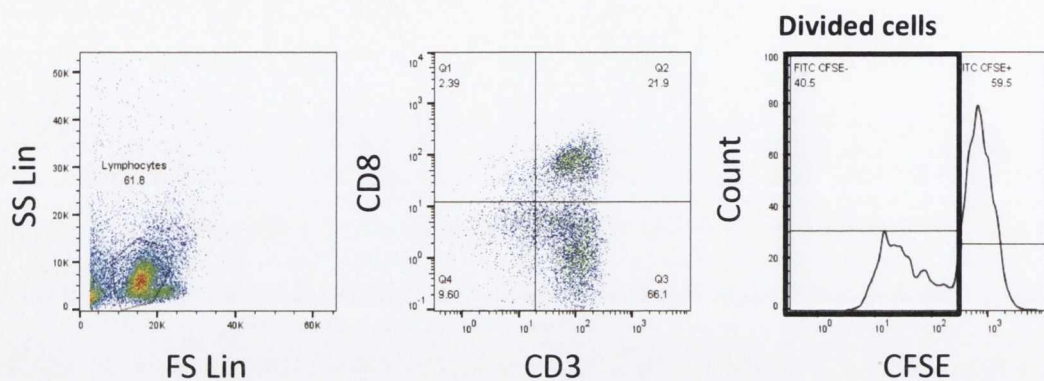


Figure 3.24: Effect of tissue microenvironment on T cell proliferation.

A) Flow plots show gating strategy used for analysis. B) Histograms show CFSE intensity before and after treatment with conditioned media.

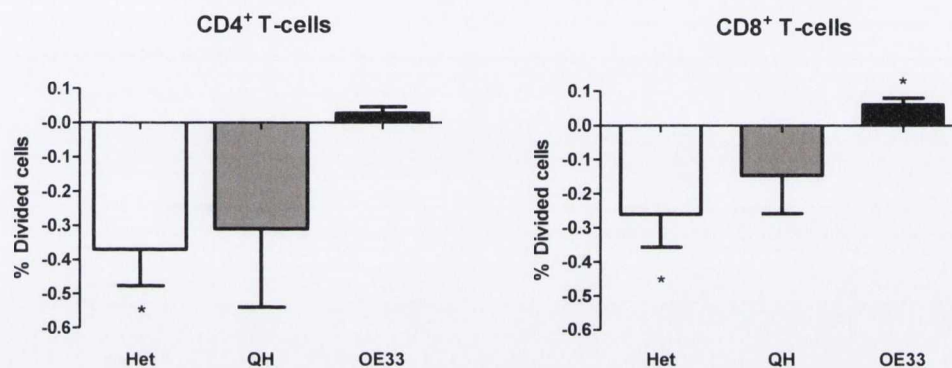


Figure 3.25: Effect of cell line supernatant on T cell proliferation.

PBMCs were isolated from blood and stimulated with PMA and ionomycin in the presence cell line supernatant. Control cells were treated with media only. Proliferation was measured by CFSE staining after 5 days in culture. A) Representative histograms showing CFSE intensity. B) Bar charts showing % dividing cells. All data is expressed as fold change in % cells divided compared to media only control $\pm$ SEM. * $p \leq 0.05$, compared to media only control, paired t test, $n=4$.

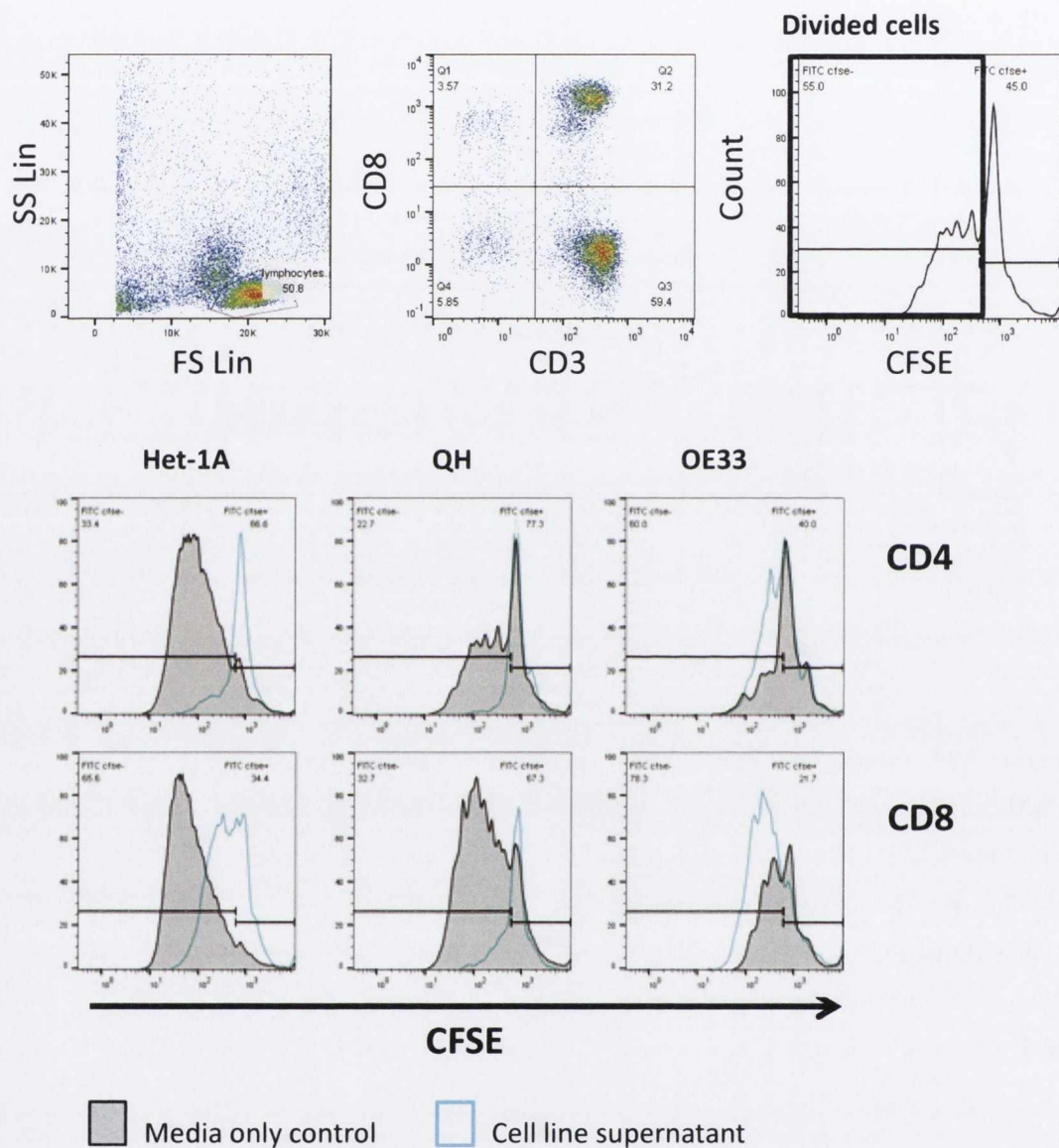


Figure 3.26: Effect of cell line supernatant on T cell proliferation.

A) Flow plots show gating strategy used for analysis. B) Histograms show CFSE intensity before and after treatment with cell line supernatant.

3.3 Discussion

The aim of this chapter was to carry out a detailed immunophenotyping of CD4⁺ and CD8⁺ T cells in oesophagitis, Barrett's and OAC tissue. While previous studies have proposed a Th1 type cytokine profile in Oesophagitis, Th2 in Barrett's and a return to Th1 again in OAC [107, 235], the actual T cell profile in the tissue has not been examined. This study directly assesses the profile of infiltrating T cells within ex-vivo tissue samples across the GORD-BO-OAC sequence and examines how secreted factors from the local tissue microenvironment shape T cell phenotype and responses.

The acute inflammatory response in oesophagitis is characterised by increased levels of proinflammatory cytokines including IL-1 β , CXCL8, and IFN- γ [72, 107]. Our data supports these studies, with higher levels of IL-2 and IFN- γ observed in oesophagitis microenvironment. We have also demonstrated that secreted factors from oesophagitis tissue significantly enhance CD8⁺ T cell proliferation, which may be induced by the higher levels of secreted IL-2 in the microenvironment. This may suggest that early in disease progression factors in the oesophageal microenvironment may promote the expansion of resident Th1 cells thus contributing to the cell mediated Th1 profile observed in oesophagitis. While this study focused on the effects of the tissue microenvironment on CD4⁺ and CD8⁺ T cells, it is likely that such secreted factors influence other resident immune cells, possibly shaping the entire immune profile in inflamed oesophagitis tissue and contributing to subsequent pathological consequences.

After prolonged periods of caustic injury to the luminal surface of the lower oesophagus due to bile reflux with high volume acid reflux, intestinal metaplasia and Barrett's oesophagus occurs in 5-13% of patients [6]. Previous studies have described a predominant Th2 cytokine profile in Barrett's oesophageal tissue [107, 235]. In the current study, analysis of the T cells within Barrett's oesophageal tissue biopsies revealed significantly higher proportions of IL-4 producing CD4⁺ T cells and elevated proportions of IL-10 expressing CD4⁺ and CD8⁺ T cells compared with normal tissue. IL-4 is a known trigger for intestinal metaplasia and is increased in BO compared to normal and oesophagitis tissue [107, 239]. Furthermore, as IL-4 inhibits Th1 responses by suppressing Th1 differentiation and by inhibiting IFN- γ production [240], the higher frequencies of IL-4 producing CD4⁺ T cells identified in this study, may play a role in

switching the balance from a Th1 response in oesophagitis to a more Th2 response in BO and in the metaplastic transformation.

This study identified significantly elevated levels of Th2 cytokines IL-10 and IL-6 in the BO microenvironment and supports previous findings of the predominantly humoral response at this disease stage [241, 242]. In one murine study, a deficiency in IL-6, was shown to halt this disease progression, with homozygous IL-6^{-/-} mice displaying a complete abolishment of the metaplastic and dysplastic phenotype whereas, heterozygous IL-6^{+/-} mice displayed diminished metaplasia with no dysplasia [45]. In oesophageal squamous cell carcinoma IL-6 has been associated with poor prognosis and inhibition of IL-6 has shown promising results, reducing radiation resistance and aggressive tumour behaviour both *in vitro* and *in vivo*, and we propose that similar studies in oesophageal adenocarcinoma may reveal new therapeutic avenues for patients [243]. Given the important role of IL-6 in driving this disease progression, we believe that inhibition of this single cytokine may provide a therapeutic benefit for patients and may prevent the metaplastic transformation to BO and subsequent tumorigenesis. Furthermore, this study demonstrated higher levels of IL-1 β in the Barrett's microenvironment; the importance of IL-1 β in the progression of GORD to BO and OAC has been demonstrated by Quante *et al.*, who overexpressed human IL-1 β in the oesophagus to create a murine model for the oesophagitis-metaplasia-adenocarcinoma sequence [45]. Similarly our results identified increased levels of GM-CSF within the BO and tumour microenvironment. GM-CSF plays a vital role in the maturation of dendritic cells and may therefore play a vital role in the activation of T cells. The tumour microenvironment however, can induce a tolerogenic phenotype in DCs [244, 245]. Soluble factors present can reduce antigen presentation capacity, reduce expression of co-stimulatory molecules and induce a tolerogenic phenotype with increased IL-10 expression and reduced IL-12 expression [244, 245]. Therefor the increase in GM-CSF seen in the OAC microenvironment in this study may suggest an increase in the maturation of these tolerogenic DCs thus impairing T cell activation.

Treatment of healthy PBMC with conditioned media from BO tissue significantly enhanced T cell activation compared with oesophagitis tissue. The proportion of T cells producing IFN- γ and TNF- α were significantly reduced, whereas the frequency of Th2

cells producing IL-4 or IL-10 remained unchanged. This reduction in Th1 type cells may be explained in part by the increased levels of IL-6 and IL-10 in the Barrett's microenvironment. This provides a mechanism whereby factors in the BO microenvironment down regulate Th1 responses in favour of a more tumour promoting Th2 profile. Therapeutically reducing IL-6 or IL-10 levels in the oesophageal microenvironment may prevent the Th2 phenotype present in BO from "carrying over" into the tumour setting and help create an environment more conducive to an effective anti-tumour immune response. Interestingly when PBMC were treated with CLS from QH (Barrett's metaplasia) cell line a similar reduction in the proportion of TNF- α and IL-12 producing cells were observed compared to media only treated cells. Again this suggests a prominent down regulation of the Th1 like response.

The establishment of an appropriate immune response in cancer has the potential to induce effective immune cell activation and accumulation at the tumour site thus leading to tumour regression [246, 247]. In OAC and oesophageal squamous cell carcinoma the presence of CD8⁺ cytotoxic lymphocytes and/or CD4⁺ T cells has been associated with improved patient prognosis [120, 248]. However, it is known that tumour infiltrating lymphocytes (TILs) from patients with advanced cancer are functionally deficient [249]. In our study, characterisation of the activation status of tumour infiltrating lymphocytes in OAC tissue revealed lower frequencies of activated CD45RO⁺ and CD69⁺ T cells. Similarly, circulating levels of CD69⁺ T cells were significantly reduced in OAC patients compared to Barrett's patients, suggesting a systemic effect on T cells resulting in reduced activation. Therefore our data suggests impaired activity and function which is likely to result in a diminished anti-tumour immune response. Studies have identified an association between the presence of activated and memory T cells within the TIL population, and overall patient survival. In head and neck squamous cell carcinoma, studies have demonstrated that higher levels of CD4⁺CD69⁺ T cells correlate with increased tumour control and longer survival [250], similarly in advanced stage ovarian cancer, the presence of CD45RO⁺ T cells were associated with improved prognosis and survival [251]. Therefore our data may suggest a diminished anti-tumour immune response. We report that levels of both pro- and anti-inflammatory cytokines were significantly higher within the OAC microenvironment compared with the normal microenvironment, and it is not exclusively Th1 as some studies have suggested [105, 106]. These cytokines include

IFN- γ , TNF- α , IL-1 β , IL-12p70, IL-10, IL-6, and IL-2, which may result in a mixed T cell profile within the tumour tissue.

Similar to results with BCM, treatment of healthy PBMC with conditioned media from OAC tumour tissue significantly reduced the proportion of Th1 T cells producing IFN- γ and TNF- α . Local secretion of IFN- γ is known to enhance anti-tumour activity, increasing antigen presentation and promoting CD8⁺ T cell proliferation, expansion and survival [252, 253]. TNF- α signalling in T cells is also critical for the priming, proliferation and recruitment of tumour specific T cells [254] and in its absence tumour immune surveillance is diminished. While secreted levels of these two cytokines were shown to be increased in the TCM, treatment with TCM reduced IFN- γ and TNF- α expression by both CD4⁺ and CD8⁺ T cells. This suggests that the effects elicited by Th2 or anti-inflammatory cytokines, such as IL-6 and IL-10, may outcompete and neutralise any potentially beneficial effects of the proinflammatory cytokines, thus impairing anti-tumour immunity.

In conclusion, this chapter for the first time characterised the T cell immunophenotype in GORD, BO and OAC. We demonstrated a Th1 profile within the oesophagitis microenvironment, shifting towards a more Th2 profile in BO, associated with an increase in IL-4 producing Th2 T cells, and secreted IL-6 and IL-10 in the microenvironment (Figure 3.27). In OAC, we demonstrate an increase in both pro and anti-inflammatory cytokines, suggesting a more mixed inflammatory profile at this disease stage, and that the frequencies of activated T cells found in OAC tissue are significantly lower than in normal or BO tissue. Our novel data suggests that secreted factors within the tissue microenvironment can initially enhance T cell proliferation in oesophagitis, potentially allowing for the expansion of resident Th1 cells thus driving the characteristic pro-inflammatory response. No alteration in T cell proliferation was observed in BO, implying that the distinct immune cell response seen at this stage may instead involve the recruitment of specific T cell subsets. Overall, secreted factors from the oesophageal microenvironment lead to lower numbers of activated T cells and altered cytokine expression, meaning it may have a detrimental impact on T cell mediated anti-tumour immunity. A greater understanding of the factors which impair T cell mediated immune responses in the local oesophageal microenvironment in OAC

may lead to the identification of novel immune based interventions to alter the tissue microenvironment and promote/augment T cell-mediated anti-tumour responses.

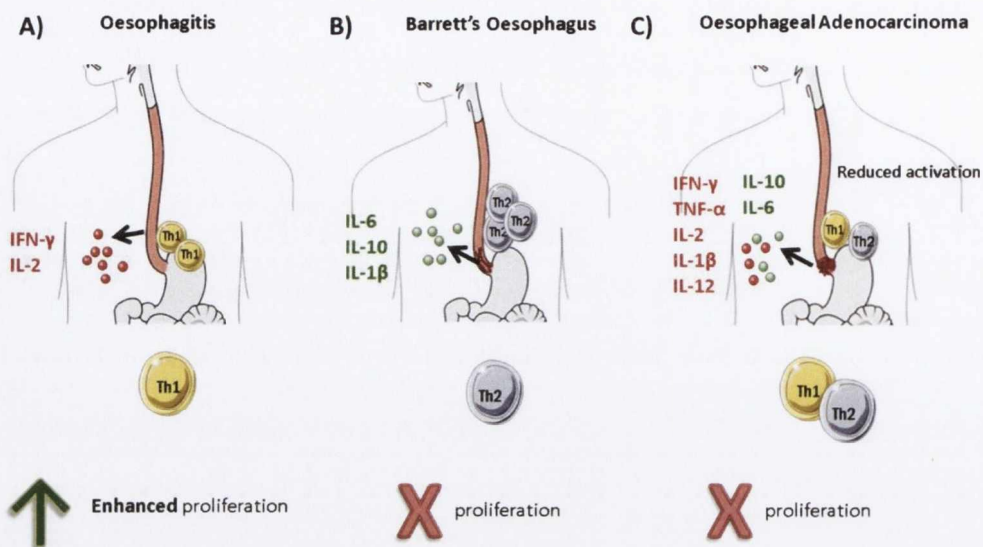


Figure 3.27: Summary of findings from chapter 3.

A) Results confirmed the presence of a Th1 response in Oesophagitis with increased levels of secreted IFN- γ and IL-2. These cytokines are likely to drive a Th1 differentiation within the oesophageal microenvironment. Importantly results demonstrated that factors in the OCM could increase T cell proliferation. B) Within the Barrett's microenvironment results demonstrated increased levels of IL-6, IL-10 and IL-1 β , which are likely to drive Th2 polarisation while inhibiting Th1 differentiation. Furthermore an increase in IL-4 and IL-10 producing Th2 cells were identified in BO tissue. C) Within OAC tissue we noted a reduction in the proportion of activated T cells, suggesting a diminished immune response. A mixed cytokine profile was observed within the OAC microenvironment; these included IFN- γ , TNF- α , IL-2, IL-1 β , IL-12, IL6 and IL-10. Factors present in the BO OAC microenvironment were found to reduce TH1 producing cells, potentially driving disease progression. T cell proliferation was not altered during BO or OAC suggesting that the immune cell profile in Barrett's and OAC tissue must be regulated by some other mechanisms.

4. Chapter 4- Results

Assessing the impact of chemokines in T cell recruitment to GORD, BO and OAC tissue.

4.1 Introduction

Results from chapter 3 supported previous findings of a predominantly Th1 and Th2 response in oesophagitis and Barrett's oesophagus (BO) respectively [72, 107, 235] and for the first time we demonstrated higher proportions of IL-4 and IL-10 producing T cells within BO tissue. This switch to a Th2 profile is not only thought to drive the metaplastic transformation in BO [113], but it is also thought to be pro-tumourigenic [123], and thus promote OAC development. While our results demonstrated that factors present in the oesophagitis microenvironment could significantly increase T cell proliferation, the same was not demonstrated in BO. Thus the accumulation of Th2 cells in BO tissue is not likely to be due to proliferation and expansion of resident T cells and may therefore be mediated through altered recruitment of T cells.

Recent studies have identified a novel mechanism, whereby exposure of oesophageal epithelial cells to refluxed acid upregulates CXCL8 expression and induces the migration of inflammatory cells to oesophageal tissue [72, 177, 178]. Thus it is suggested that chemokines, rather than caustic injury, mediate inflammation damaging the oesophageal mucosa in GORD. Elevated levels of CCL2, CCL5 and CXCL8 have all been reported in oesophagitis tissue compared to healthy tissue and have been shown to correlate with level of severity [179]. However, despite their importance in the initiation of inflammation in the oesophagus, the chemokine profile in the oesophagitis-metaplasia-carcinoma sequence has not been studied in detail to date, nor have T cell specific chemokines been identified.

Chemokines are chemotactic cytokines that promote the directional migration of cells through a concentration gradient. The expression of chemokine receptors on T cells has been demonstrated to regulate T cell trafficking, and the differential chemokine receptor expression on T cells has been shown to be capable of distinguishing distinct T cell subsets. Naïve T cells and memory T cells express the chemokine receptor CCR7 which binds to CCL21 and allows for their circulation through blood and lymph nodes [75, 166]. Following activation, T cells rapidly downregulate CCR7 and increase expression of CXCR3 [168]. Furthermore, T helper subsets can be identified based on chemokine expression. Th1 cells are known to predominantly express CCR5, CXCR3 and CXCR6 [255, 256]. CCR5 is a receptor for the inflammatory chemokines CCL5, CCL3 and CCL4. CXCR3 is a receptor for CXCL10 and CXCL11, both of which are

induced by IFN- γ [172]. Th2 cells, on the other hand primarily express CCR3 and following activation CCR4 [255, 256]. CCR4 binds to the chemokines CCL17 and CCL22 and is also expressed on regulatory t cells. CCR3 binds to eotaxin (CCL11), eotaxin-2 (CCL12), CCL5, MCP-2 (CCL8), MCP-3 (CCL7) and CCL13 and is selectively expressed on IL-4 producing Th2 cells [157, 170]. CCR6 is the only known receptor for CCL20 and is found to be expressed on Th17 cells and memory T cells.

Studies have reported that CCL17 and CCL22 play a role in the recruitment of regulatory T cells to tumour tissue in gastric cancer and oesophageal squamous cell carcinoma, thus impairing anti-tumour immunity [182, 183]. However to date no studies have identified chemokine pathways at play in T cell recruitment to OAC tissue or its precursor lesion BO. It is possible that an altered chemokine profile within the oesophageal microenvironment may play a role during the GORD-BO-OAC progression, and may promote the recruitment of distinct T cell subsets to oesophageal tissue. Thus identifying chemokine pathways at play may allow for therapeutic interventions to alter T cell trafficking and modify the immune cell infiltrate.

Immunotherapeutic targeting of the CCR4 receptor has proved successful in reducing the infiltrating Treg population in numerous cancers. Mogamulizumab is a humanised mAB against CCR4 that has been approved for the treatment of adult T cell leukaemia [257, 258]. It has been shown to selectively deplete infiltrating Treg populations and thus can evoke an anti-tumour immune response. It is possible that similar strategies may be beneficial in reversing the Th2 cell accumulation in BO tissue and may therefore prevent disease progression.

This chapter examines the hypothesis that the chemokines present in the local oesophageal microenvironment may contribute to the distinct T cell polarisation observed at each disease stage. The specific aims were to:

- Characterise the chemokine profile in patient serum and within the oesophageal microenvironment at each disease stage
- Phenotype T cell chemokine receptor expression in blood and oesophageal tissue, in order to identify the cells likely to respond to chemokines in the oesophageal microenvironment at each disease stage

- Examine the impact of chemokines in the oesophageal microenvironment on T cell migration
- Explore the potential for chemokine receptor blockade as potential immunotherapeutic strategy to alter predominant T cell subsets at each disease stage

4.2 Results

4.2.1 Analysis of chemokine receptors in circulating T cells

A detailed analysis of chemokine receptor expression on circulating T cells was carried out to identify the chemokine pathways that may play a role in T cell recruitment to oesophageal tissue. Whole blood staining was carried out on normal, oesophagitis, BO, and OAC bloods for CD3, CD4, CD8, CCR5, CXCR3, CCR4, CCR3 and CCR6. CCR5 and CXCR3 are predominantly found on Th1 cells; CCR3 and CCR4 on Th2; and CCR6 is found predominantly on Th17 and Treg cells [255, 256]. Therefore this panel design was chosen to identify the potential T cell subsets that may be recruited to oesophageal tissue.

The predominant chemokine receptors expressed on Th1 cells are CXCR3 and CCR5, which bind CXCL10/CXCL11 and CCL3/MIP-1 β respectively [172]. Our data demonstrated significantly higher levels of CD4⁺CXCR3⁺ cells in oesophagitis patients ($38.7 \pm 4.32\%$) compared to healthy controls ($17.29 \pm 4.93\%$, $p < 0.05$) (Figure 4.1), suggesting Th1 inflammatory cells may predominate at this stage. Conversely, OAC patients displayed significantly lower levels of CD4⁺CXCR3⁺ ($1.79 \pm 0.28\%$) compared to oesophagitis ($38.7 \pm 4.32\%$, $p < 0.001$) and Barrett's patients ($30.11 \pm 2.42\%$, $p < 0.001$). Similarly in the CD8⁺ population OAC patient displayed significantly lower levels of CXCR3⁺ cells ($1.36 \pm 0.28\%$) compared to normal ($35.08 \pm 8.80\%$, $p < 0.05$), oesophagitis ($59.83 \pm 5.17\%$, $p < 0.01$) and Barrett's patients ($45.053 \pm 3.33\%$, $p < 0.01$), suggesting a diminished Th1 response in OAC. Levels of CCR5 expressing cells were not significantly different between patient groups (Figure 4.2).

Th2 cells are known to primarily express CCR3 and CCR4. Among the chemokines they respond to are CCL5 and CCL3/CCL4 and CCL17 respectively. The percentage of cells expressing CCR4 was found to be similar across all patient groups (Figure 4.3). Barrett's patients demonstrated significantly higher levels of CD4⁺ and CD8⁺ CCR3 cells (CD4: $6.57 \pm 4.74\%$; CD8: $4.01 \pm 0.88\%$) compared to normal (CD4: $0.45 \pm 0.16\%$, $p < 0.01$; CD8: $0.91 \pm 0.45\%$, $p < 0.05$), suggesting a systemic bias to Th2 cells in BO blood. Similarly OAC patient displayed significantly higher levels of CD8⁺CCR3⁺ ($4.69 \pm 0.73\%$) compared to normal ($0.91 \pm 0.45\%$, $p < 0.05$) (Figure 4.4) again suggesting a predominantly Th2 T cell population in OAC blood.

The chemokine receptor CCR6 responds exclusively to CCL20 and is found on Th17 cells. Our data demonstrates significantly lower levels of circulating CD4⁺ and CD8⁺CCR6⁺ cells in OAC patients (CD4: 0.43 ± 0.84%; CD8: 17.28 ± 1.36%) compared to normal (CD4: 13.6 ± 4.11%, p<0.05; CD8: 32.41 ± 4.63%, p<0.05), oesophagitis (CD4: 16.2 ± 13.63%, p<0.05; CD8 NS) and Barrett's patients (CD4: 16.10 ± 3.371%, p<0.01; CD8: NS) (Figure 4.5).

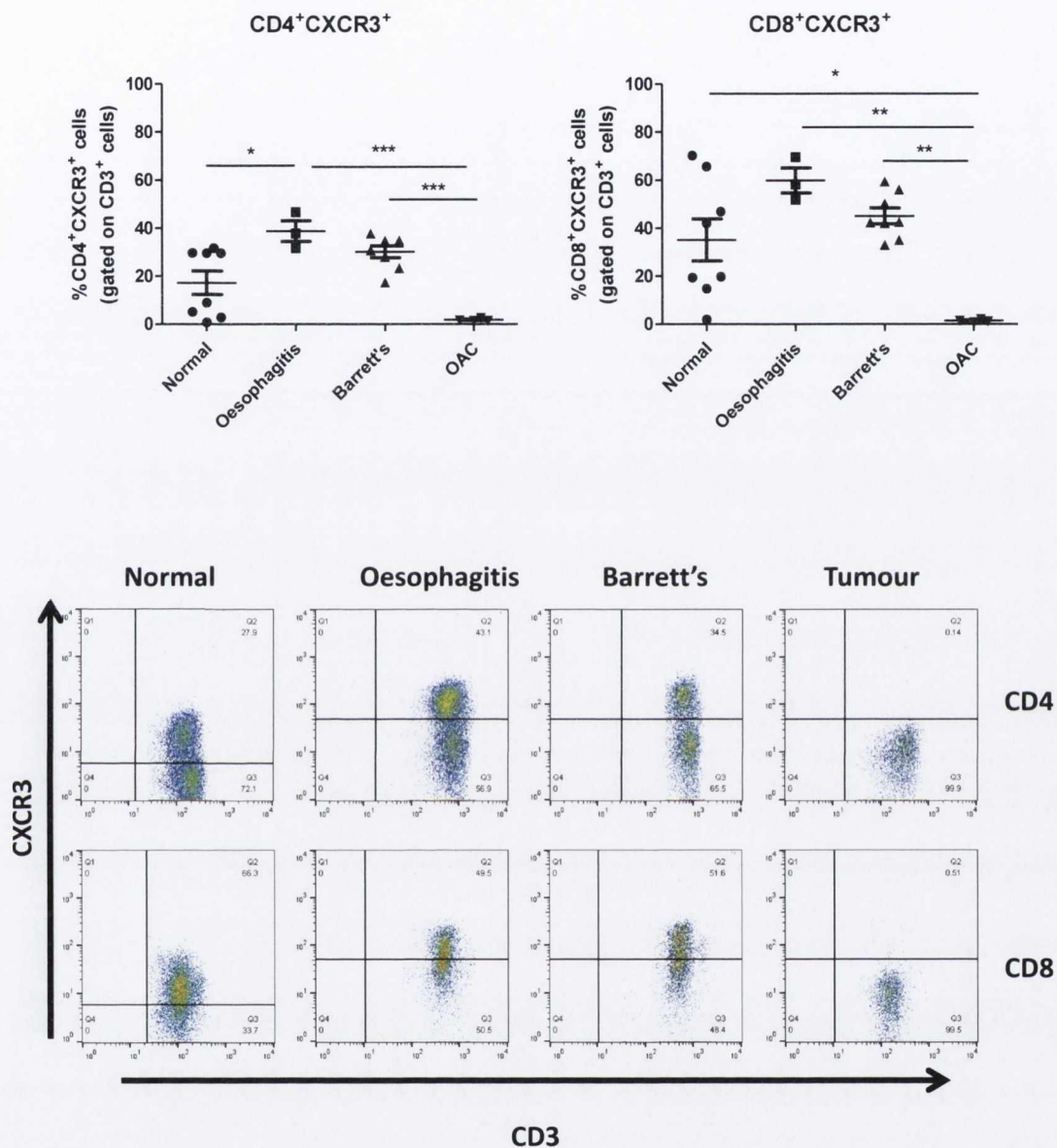


Figure 4.1: CXCR3 expression on circulating T cells.

Whole blood from healthy control, oesophagitis, Barrett's and OAC patients was stained for CD3, CD4, CD8 and CXCR3 by flow cytometry. A) Scatter plot show percentage of CD4⁺ or CD8⁺ T cells expressing CXCR3. B) Flow plots show CXCR3 expression on CD4⁺ and CD8⁺ cells in normal, oesophagitis, Barrett's and OAC patient blood. All data is presented as mean $\pm$ SEM, n=3-8, *p $\leq$ 0.05, **p $\leq$ 0.01, ***p $\leq$ 0.001 One way ANOVA, Dunns post-test. N= (Normal =8, Oesophagitis=3, BO=8, OAC=5).

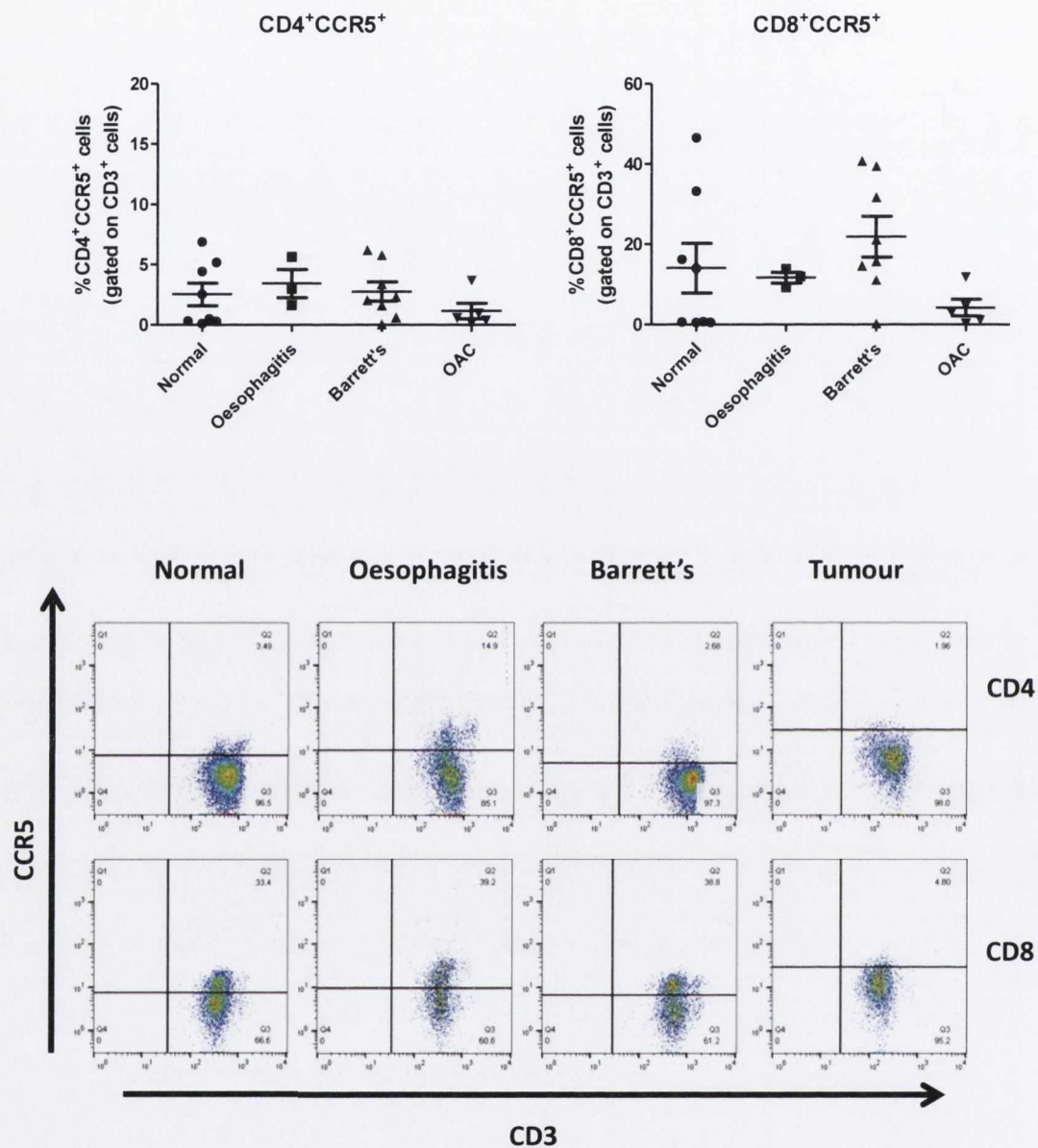


Figure 4.2: CCR5 expression on circulating T cells.

Whole blood from healthy control, oesophagitis, Barrett's and OAC patients was stained for CD3, CD4, CD8 and CCR5 by flow cytometry. A) Scatter plot show percentage of CD4⁺ or CD8⁺ T cells expressing CCR5. B) Flow plots show CCR5 expression on CD4⁺ and CD8⁺ cells in normal, oesophagitis, Barrett's and OAC patient blood. All data is presented as mean $\pm$ SEM, n=3-8, One way ANOVA, Dunns post-test. N= (Normal=8, Oesophagitis=3, BO=8, OAC=5).

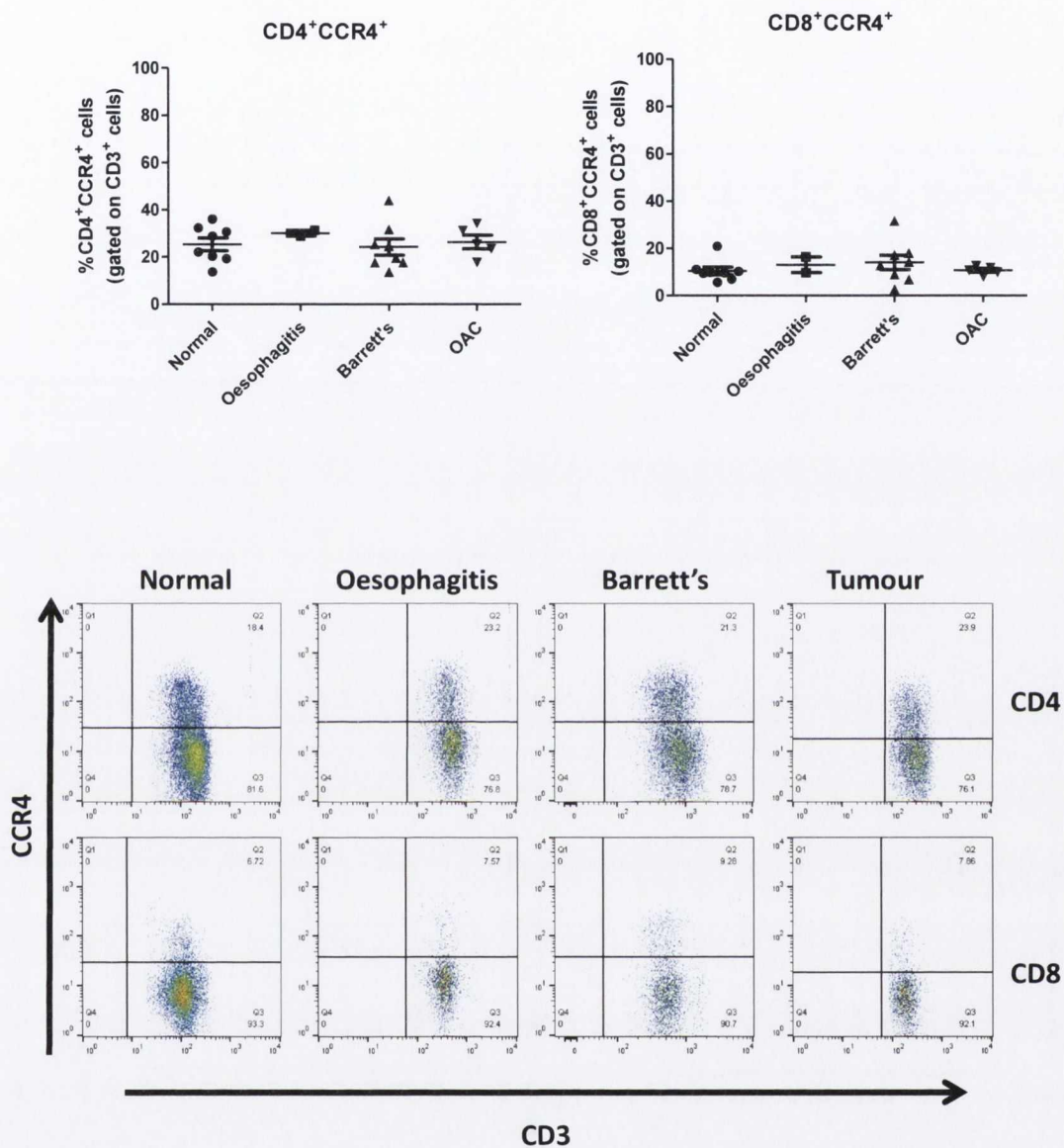


Figure 4.3: CCR4 expression on circulating T cells.

Whole blood from healthy control, oesophagitis, Barrett's and OAC patients was stained for CD3, CD4, CD8 and CCR4 by flow cytometry. A) Scatter plot show percentage of CD4⁺ or CD8⁺ T cells expressing CCR4. B) Flow plots show CCR4 expression on CD4⁺ and CD8⁺ cells in normal, oesophagitis, Barrett's and OAC patient blood. All data is presented as mean $\pm$ SEM, n=3-8, One way ANOVA, Dunns post-test. N= (Normal =8, Oesophagitis=3, BO=8, OAC=5).

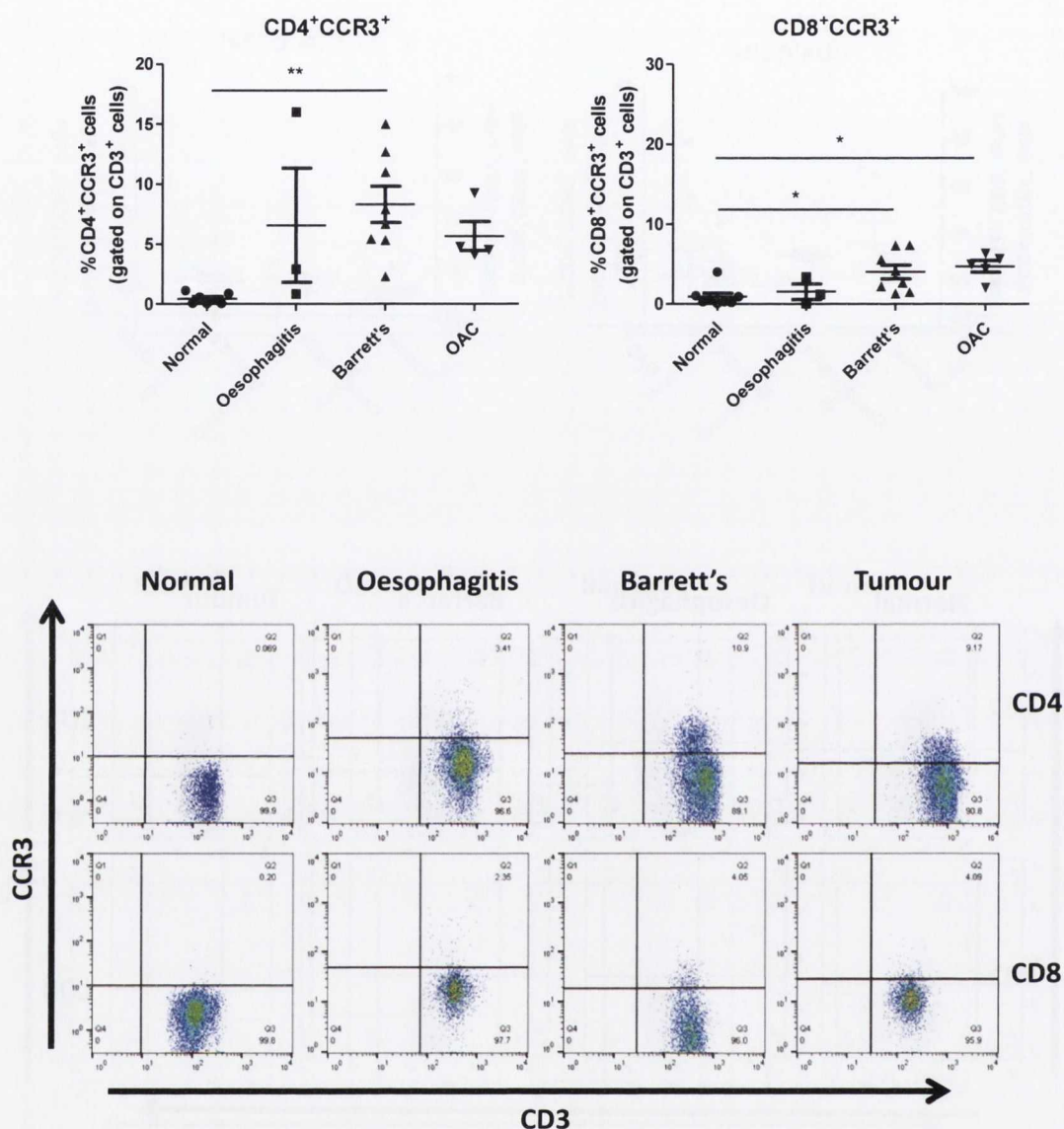


Figure 4.4: CCR3 expression on circulating T cells.

Whole blood from healthy control, oesophagitis, Barrett's and OAC patients was stained for CD3, CD4, CD8 and CCR3 by flow cytometry. A) Scatter plot show percentage of CD4⁺ or CD8⁺ T cells expressing CCR3. B) Flow plots show CCR3 expression on CD4⁺ and CD8⁺ cells in normal, oesophagitis, Barrett's and OAC patient blood. All data is presented as mean $\pm$ SEM, n=3-8, *p $\leq$ 0.05, **p $\leq$ 0.01, One way ANOVA, Dunns post-test. N= (Normal =8, Oesophsitis=3, BO=8, OAC=5).

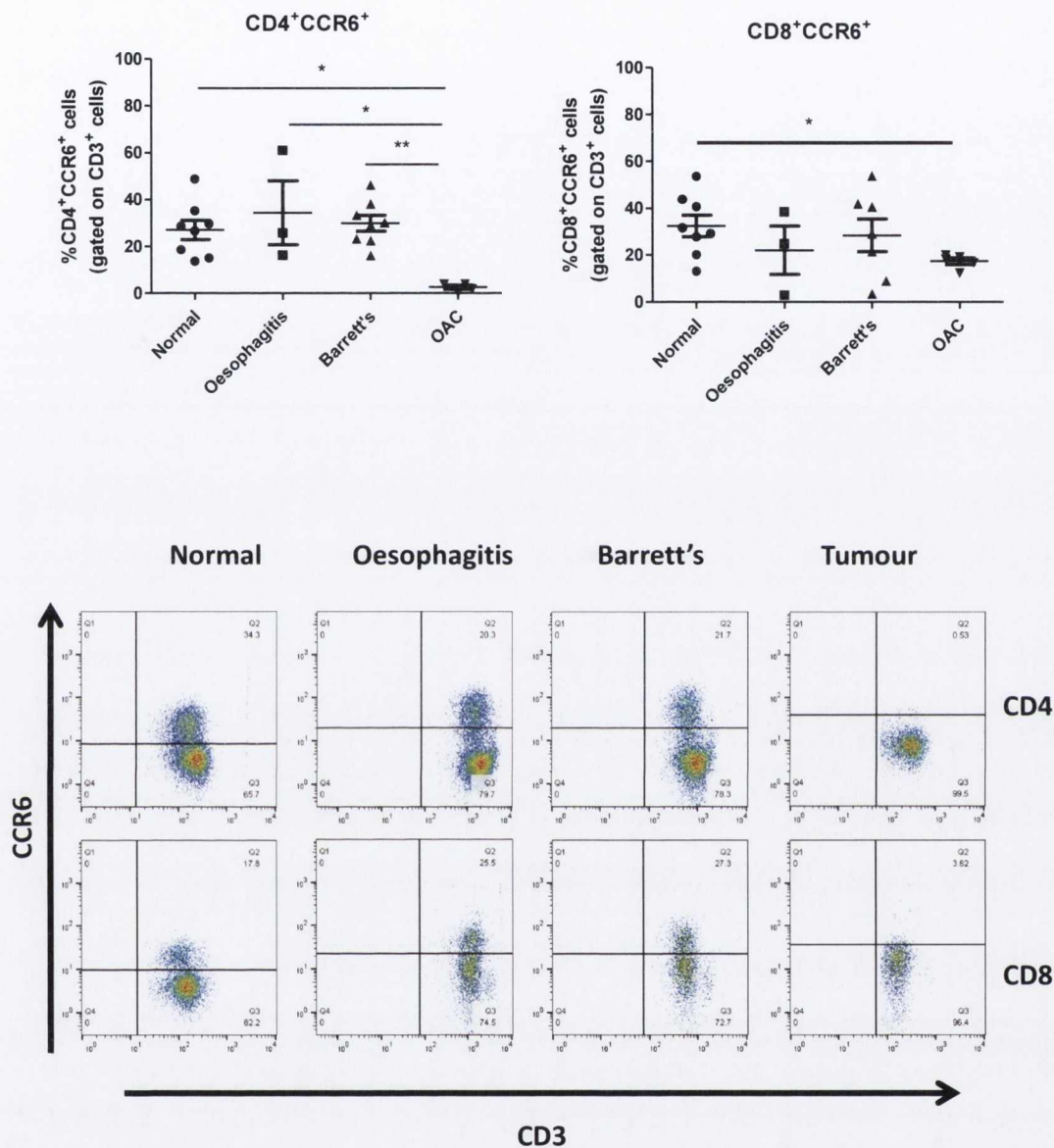


Figure 4.5: CCR6 expression on circulating T cells.

Whole blood from healthy control, oesophagitis, Barrett's and OAC patients was stained for CD3, CD4, CD8 and CCR6 by flow cytometry. A) Scatter plot show percentage of CD4⁺ or CD8⁺ T cells expressing CCR6. B) Flow plots show CCR6 expression on CD4⁺ and CD8⁺ cells in normal, oesophagitis, Barrett's and OAC patient blood. All data is presented as mean $\pm$ SEM, n=3-8, *p $\leq$ 0.05, **p $\leq$ 0.01, One way ANOVA, Dunns post-test. N= (Normal =8, Oesophagitis=3, BO=8, OAC=5).

4.2.2 Analysis of chemokines in patient serum samples

In order to determine the likelihood of T cell retention in blood, levels of chemokines were measured in patient serum. Levels of chemokines were measured in serum samples from healthy donors, oesophagitis, Barrett's, and OAC patients. CXCL10 is a chemokine that signals through the CXCR3 receptor and selectively recruits Th1 lymphocytes and monocytes. Results demonstrated significantly lower levels of CXCL10 in Barrett's ($154.2 \pm 55.36\text{pg/ml}$) compared to normal serum ($417.8 \pm 92.17\text{pg/ml}$, $p<0.05$) (Figure 4.6). CCL3 and CCL4 are known to signal through the receptors CCR4 and CCR5, recruiting macrophages, dendritic cells and T cells. Significantly lower levels of CCL3 were demonstrated in oesophagitis serum ($11.70 \pm 2.13\text{pg/ml}$) compared to normal ($27.64 \pm 2.17\text{pg/ml}$, $p<0.01$), with a similar reduction observed in serum from Barrett's patients ($9.31 \pm 0.6\text{pg/ml}$) compared to normal ($27.64 \pm 2.17\text{pg/ml}$, $p<0.01$) and OAC ($23.33 \pm 2.306\text{pg/ml}$) (Figure 4.6). CCL4 demonstrated a similar trend, with lower levels observed in oesophagitis and Barrett's serum (Oes: $51.63 \pm 4.036\text{pg/ml}$; BO: $52.51 \pm 4.036\text{pg/ml}$) compared to normal ($134.2 \pm 18.25\text{pg}$, $p<0.01$) and OAC serum ($125.5 \pm 19.11\text{pg}$, $p<0.05$) (Figure 4.6). CCL17 is a potent Th2 chemokine that binds to the receptor CCR4, selectively recruiting Th2 T cells. Level of CCL17 were significantly lower in serum from Barrett's patients ($127 \pm 12.02\text{pg/ml}$) compared to normal ($457.6 \pm 100.8\text{pg/ml}$, $p<0.01$) and OAC ($437.6 \pm 83.58\text{pg/ml}$, $p<0.01$) (Figure 4.7). Similarly levels of CCL22, CCL2 and CCL13 were all reduced in serum from oesophagitis and Barrett's patients compared to normal and OAC (Figure 4.7). CCL20 was found at very low levels in the serum and although not significant was reduced in Barrett's ($0.03 \pm 0.01\text{pg/ml}$) and OAC ($0.05 \pm 0.01\text{pg/ml}$) serum compared to normal ($0.61 \pm 0.5\text{pg/ml}$, ns) and oesophagitis ($0.82 \pm 0.44\text{pg/ml}$, ns) (Figure 4.7). Levels of CXCL11, CCL5, CX3CL1 and CXCL8 were similar across all groups with no significant differences noted (Figure 4.7). Overall, serum from oesophagitis patients demonstrated significantly lower levels of CCL3, MIP-1 β , CCL22, and CCL2 compared to normal patients; while serum from Barrett's patients displayed significantly lower levels of CXCL10, CCL3, MIP-1 β , CCL17, CCL22, CCL2 and CCL13 compared to normal patients. This suggests that compared to the bloods from normal and OAC patients, there is a reduced ability to retain associated T cells in circulation, thus if these chemokines are present in the oesophageal microenvironment, it could allow for T cell migration to diseased tissue

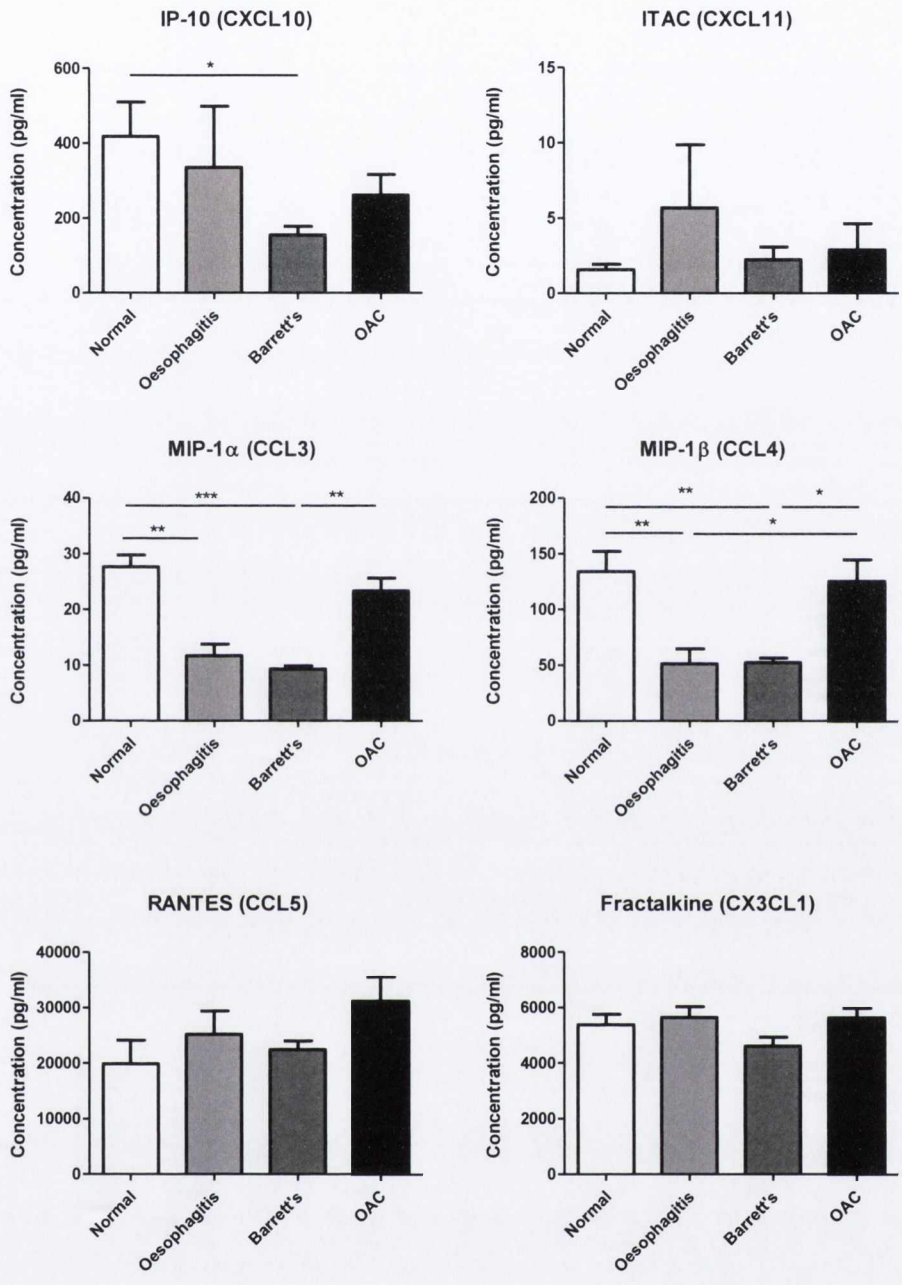


Figure 4.6: Circulating chemokine levels in patient serum.

Serum was collected from healthy controls, oesophagitis patients, BO and OAC patients and chemokine levels were measured using MSD multiplex ELISAs. All data is presented as mean $\pm$ SEM, * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, T test (Mann Whitney), $n=8-10$.

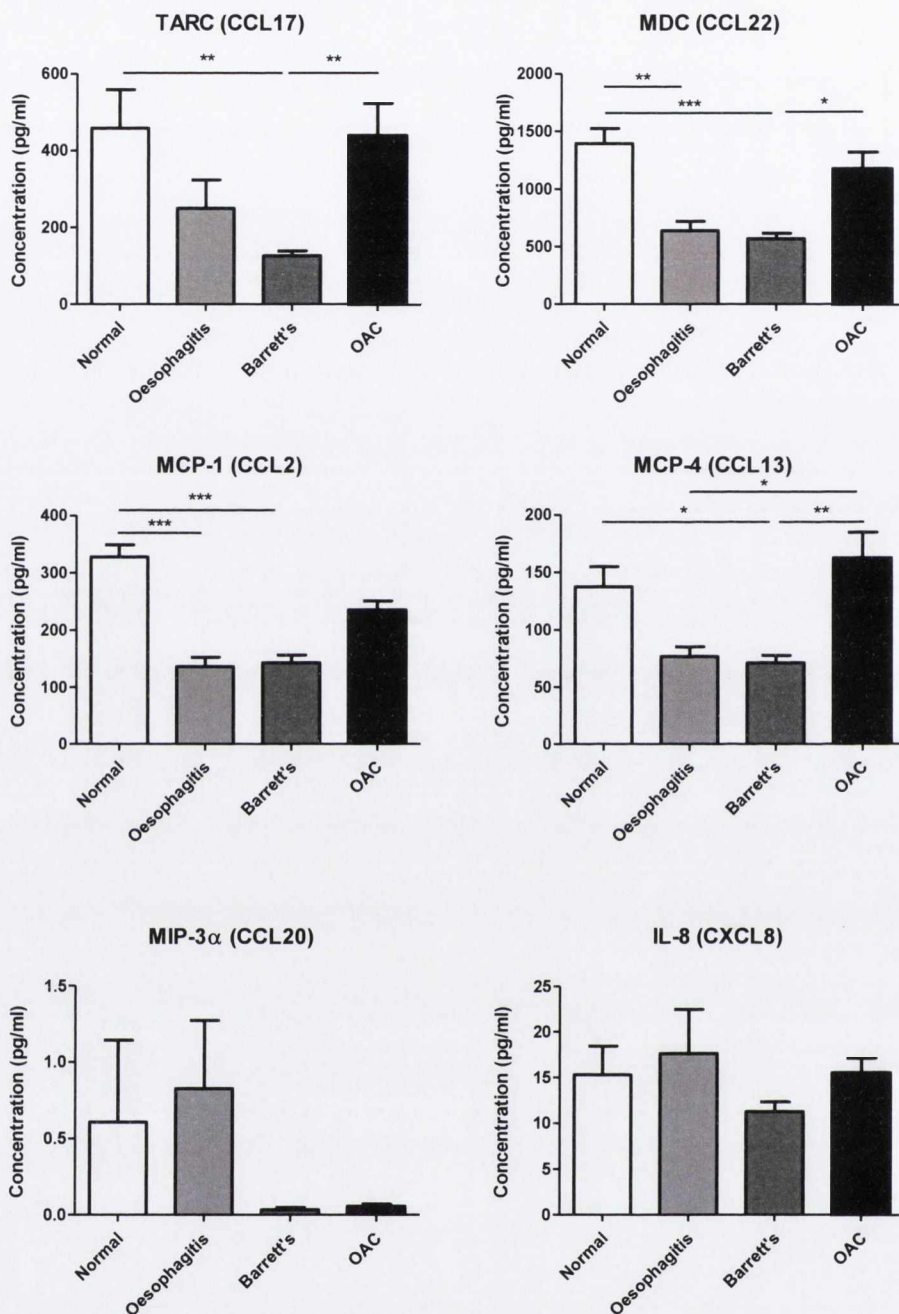


Figure 4.7: Circulating chemokine levels in patient serum.

Serum was collected from healthy controls, oesophagitis, BO and OAC patients and chemokine levels were measured using MSD multiplex ELISAs. All data is presented as mean $\pm$ SEM, * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$ T test (Mann Whitney), $n=8-10$.

4.2.3 Analysis of chemokine receptor expression on oesophageal infiltrating T cells

In order to determine whether chemokine/chemokine receptor interactions may participate in altering the immune cell populations in the GORD-BO-OAC sequence, a detailed analysis of chemokine receptor expression on T cells within oesophageal tissue was carried out. Oesophageal biopsies from normal, oesophagitis, BO and OAC tissue were enzymatically digested and stained for CD3, CD4, CD8, CCR5, CXCR3, CCR4, CCR3 and CCR6. CXCR3 and CCR5 are predominantly expressed on Th1 cells and our data demonstrated low levels of CD4⁺ and CD8⁺ cells expressing these markers across the disease sequence (Figure 4.8 and Figure 4.9). However significantly higher proportions of CD4⁺CCR5⁺ cells were observed within BO tissue ($45.83 \pm 4.64\%$) compared to normal tissue ($14.26 \pm 3.87\%$)(Figure 4.8). Th2 cells are known to primarily express CCR3 and CCR4 and again our data demonstrated expression of these markers on CD4⁺ and CD8⁺ cells infiltrating normal, oesophagitis, BO and OAC tissue. No significant differences were observed in the proportions of cells expressing CCR4 or CCR3 (Figure 4.10 and Figure 4.11). The CCR6 receptor is highly expressed on Th17 cells, our data showed a significant reduction in the proportion of CD4⁺CCR6⁺ cells within tumour tissue ($0.75 \pm 0.21\%$) compared to normal ($3.86 \pm 0.75\%$)(Figure 4.12).

4.2.4 Analysis of chemokines in the local oesophageal microenvironment

To assess whether chemokines in the local oesophageal microenvironment could recruit specific T cell subsets, oesophageal biopsies were cultured to generate conditioned media (CM); normal (NCM), oesophagitis (OCM), Barrett's (BCM) and tumour (TCM). Levels of chemokines known to recruit distinct T cell subsets were measured by MSD ELISAs. Higher levels of CXCL10 were demonstrated in TCM ($9.38 \pm 2.57\text{pg}/\mu\text{g}$) compared to BCM ($1.99 \pm 0.98\text{pg}/\mu\text{g}$, $p < 0.05$) (Figure 4.13). Interestingly, given that oesophagitis has been described as having a Th1 response, this Th1 recruiting chemokine appears to be lower in OCM ($1.94 \pm 1.16\text{pg}/\mu\text{g}$) compared with normal ($11.43 \pm 7.75\text{pg}/\mu\text{g}$, ns) conditioned media, suggesting other chemokines may be responsible for the Th1 profile. CXCL11 similarly signals through CXCR3, however levels were found to be unaltered across the disease sequence (Figure 4.13).

CCL5 signals through the CCR5 and CCR3 receptor and has been reported to be higher in oesophagitis tissue compared to healthy tissue [179]. It is a potent chemokine and is chemoattracts memory T cells and monocytes. CCL5 was found to be increased in TCM ($228.2 \pm 131.1\text{pg}/\mu\text{g}$) compared to NCM ($12.62 \pm 5.57\text{pg}/\mu\text{g}$, $p < 0.05$). Although not significant, an increase was also noted within the OCM ($63.24 \pm 46.01\text{pg}/\mu\text{g}$) compared to NCM ($12.62 \pm 5.57\text{pg}/\mu\text{g}$) (Figure 4.13). Similar results were seen with CCL3 and CCL4 although results did not reach significance. Higher levels of these 2 chemokines were observed in TCM (CCL3: $229.3 \pm 127.2\text{pg}/\mu\text{g}$; MIP-1 β : $155.3 \pm 78.11\text{pg}/\mu\text{g}$) compared to NCM (CCL3: $21.01 \pm 10.69\text{pg}/\mu\text{g}$; MIP-1 β : $7.6 \pm 3.25\text{pg}/\mu\text{g}$) (Figure 4.13), suggesting a mechanism for recruitment of Th1 CCR5⁺ cells to the OAC microenvironment.

CX3CL1 signals through the CX3CR1 receptors and potently attracts Th1 cells, CTLs, monocytes and NK cells. While no significant differences were observed in levels of CX3CL1 in the conditioned media, levels steadily increased moving from NCM ($3.39 \pm 0.8\text{pg}/\mu\text{g}$) to OCM ($5.41 \pm 2.3\text{pg}/\mu\text{g}$), BCM ($11.80 \pm 5.6\text{pg}/\mu\text{g}$) and TCM ($15.55 \pm 7.08\text{pg}/\mu\text{g}$) (Figure 4.14).

While CXCL8 has previously been reported to be increase in oesophagitis tissue, our data showed no difference in CXCL8 levels in OCM ($2257 \pm 415\text{pg}/\mu\text{g}$) compared to NCM ($1500 \pm 333\text{pg}/\mu\text{g}$). However within BCM ($8519 \pm 1694\text{pg}/\mu\text{g}$) and TCM ($21096 \pm 4963\text{pg}/\mu\text{g}$) there was a significant increase compared to normal conditioned media (Figure 4.14), suggesting CXCL8 may be responsible for T cell recruitment to BO and OAC tissue. CCL20 binds exclusively to the CCR6 receptor and was found to be significantly increased in TCM ($162.7 \pm 72.26\text{pg}/\mu\text{g}$) compared to BCM ($9.33 \pm 4.48\text{pg}/\mu\text{g}$) (Figure 4.14). No significant differences were observed in levels of CCL17, CCL22, CCL2 or CCL13.

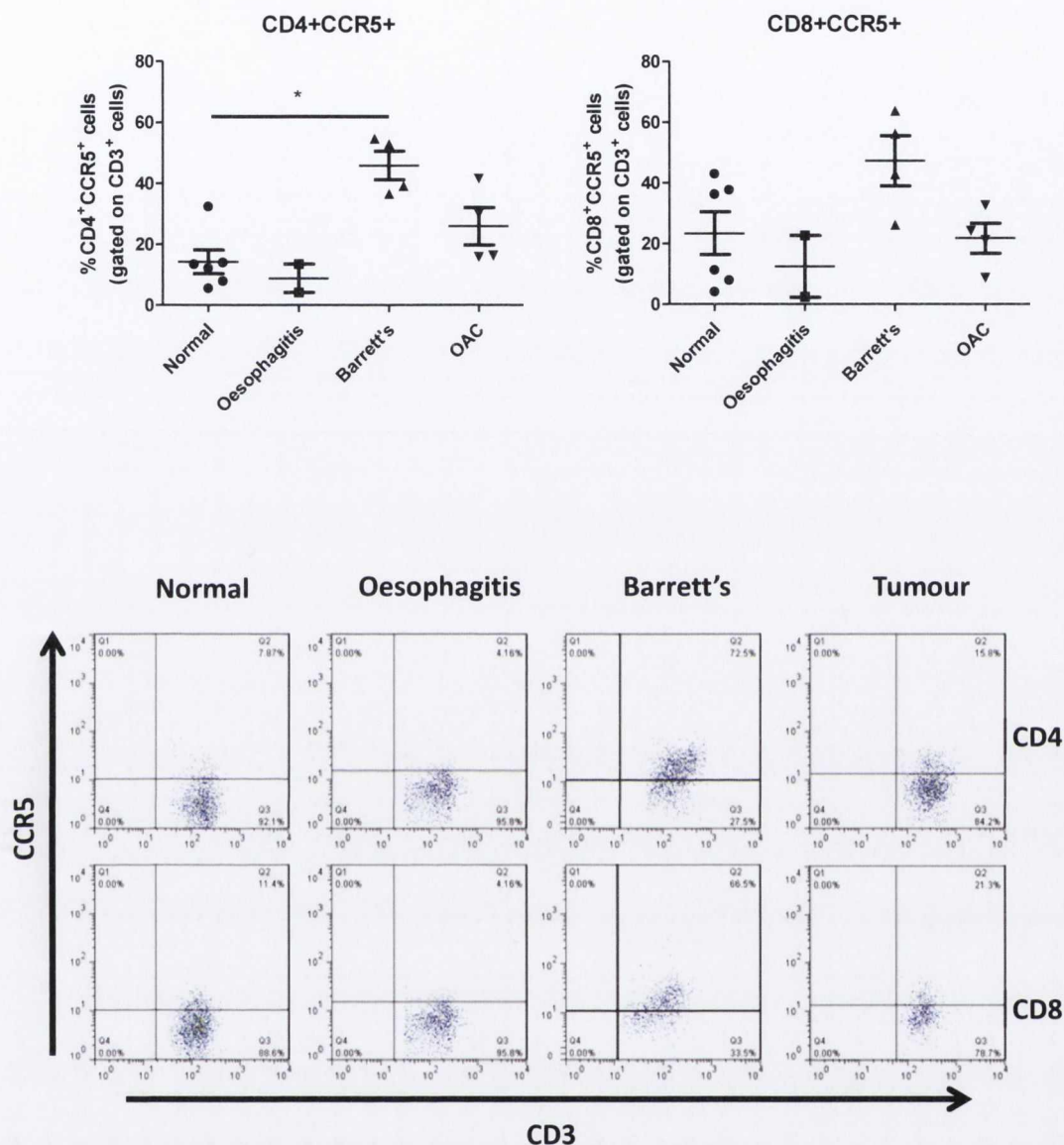


Figure 4.8: CCR5 expression on oesophageal infiltrating T cells.

Oesophageal biopsies were enzymatically digested and immunophenotyped by flow cytometry. Expression of CD3, CD4, CD8 and CCR5 were measured. A) Scatter plot showing percentage of CD4⁺ or CD8⁺ T cells expressing CCR5. B) Flow plots show CCR5 expression on CD4⁺ and CD8⁺ cells within normal, oesophagitis, Barrett's and tumour tissue. All data is presented as mean $\pm$ SEM, n=2-6, *p<0.05, One way ANOVA, Dunns post-test. N= (Normal =6, Oes=2 BO=4, OAC=4).

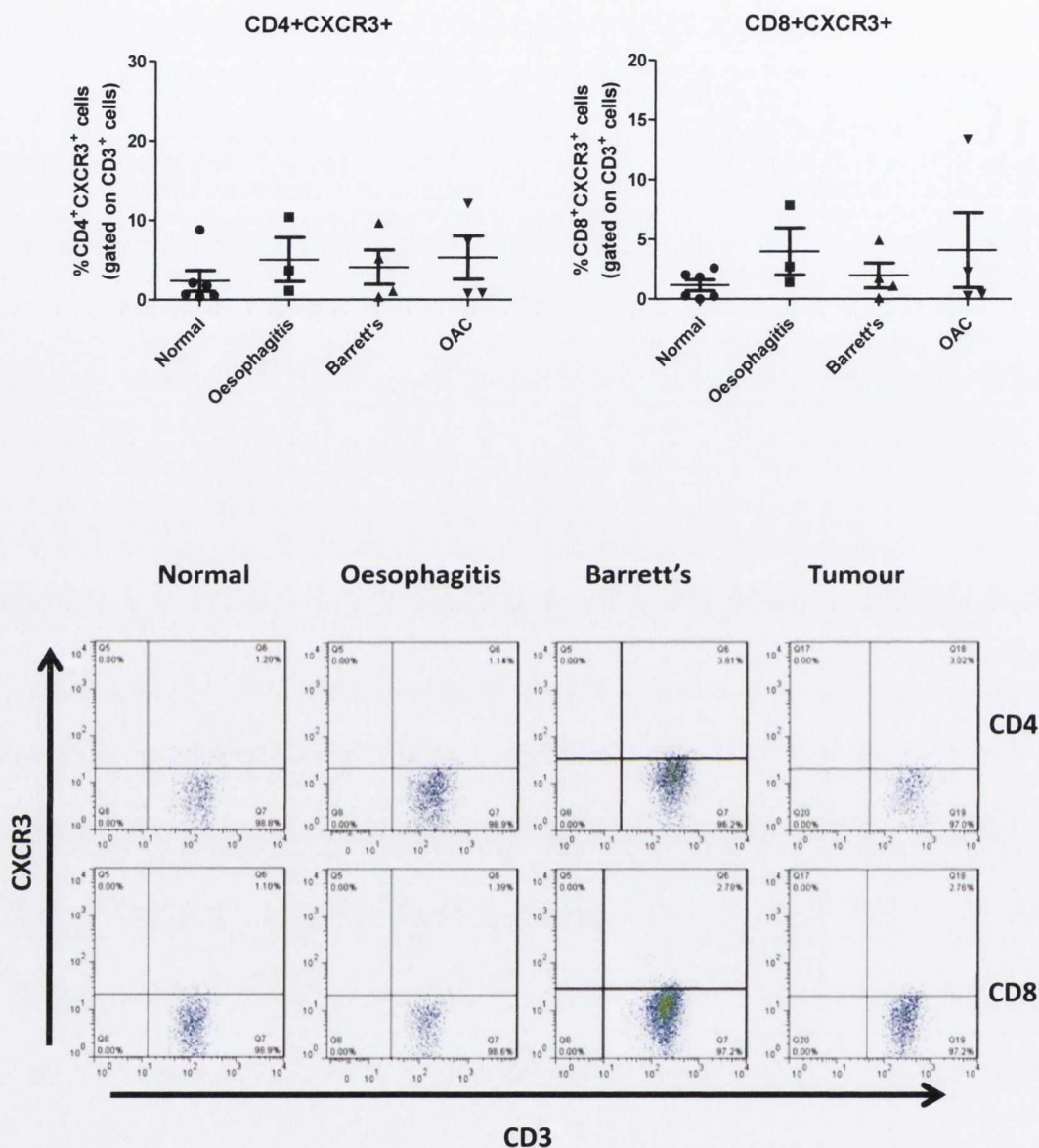


Figure 4.9: CXCR3 expression on oesophageal infiltrating T cells.

Oesophageal biopsies were enzymatically digested and immunophenotyped by flow cytometry. Expression of CD3, CD4, CD8 and CXCR3 were measured. A) Scatter plot showing percentage of CD4⁺ or CD8⁺ T cells expressing CXCR3. B) Flow plots show CXCR3 expression on CD4⁺ and CD8⁺ cells within normal, oesophagitis, Barrett's and tumour tissue. All data is presented as mean $\pm$ SEM, n=3-6, One way ANOVA, Dunns post-test. N= (Normal =6, Oes=3 BO=4, OAC=4).

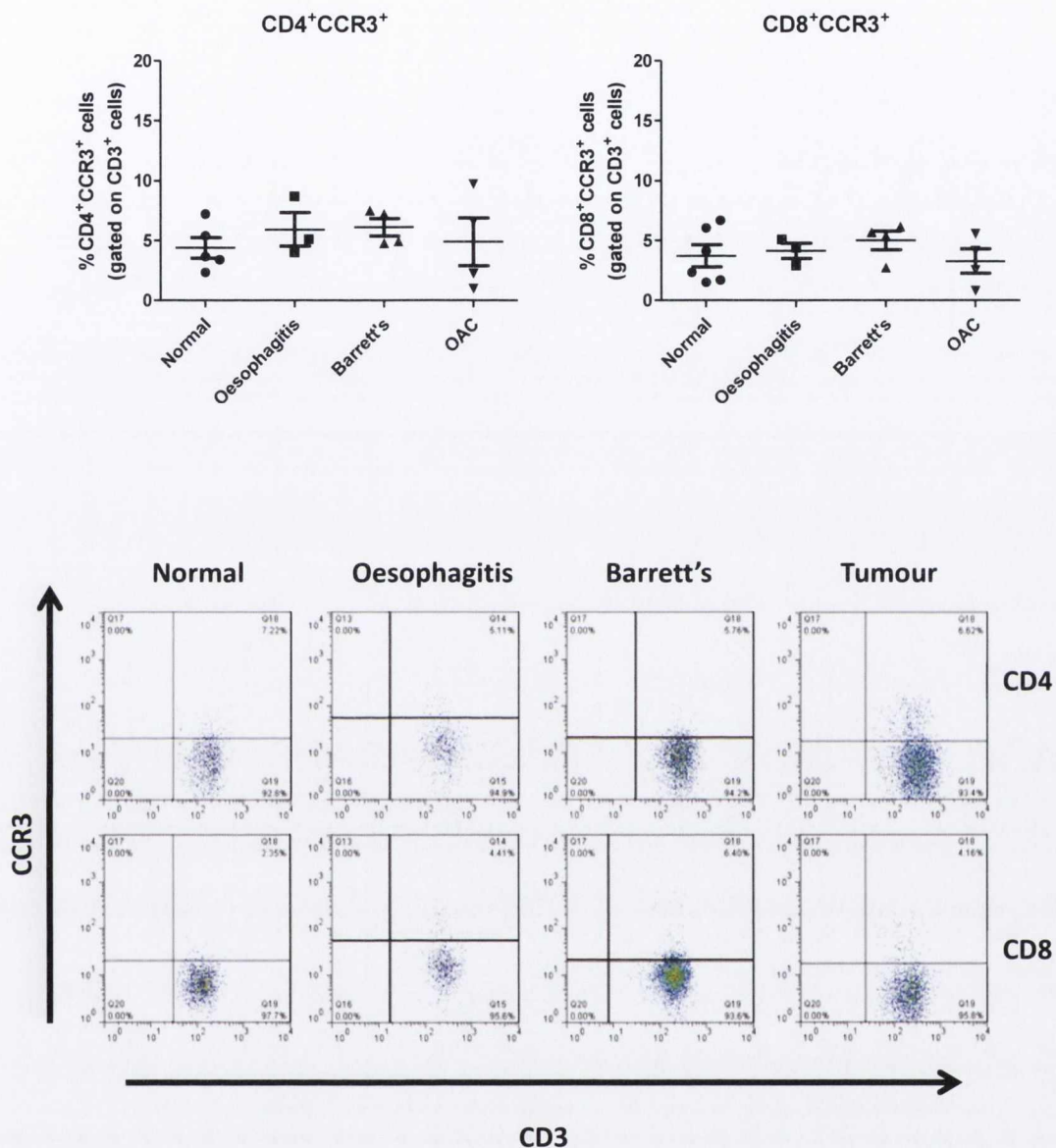


Figure 4.10: CCR3 expression on oesophageal infiltrating T cells.

Oesophageal biopsies were enzymatically digested and immunophenotyped by flow cytometry. Expression of CD3, CD4, CD8 and CCR3 were measured. A) Scatter plot showing percentage of CD4⁺ or CD8⁺ T cells expressing CCR3. B) Flow plots show CCR3 expression on CD4⁺ and CD8⁺ cells within normal, oesophagitis, Barrett's and tumour tissue. All data is presented as mean $\pm$ SEM, n=3-6, *p $\leq$ 0.05, One way ANOVA, Dunns post-test. N= (Normal =6, Oes=3 BO=4, OAC=4).

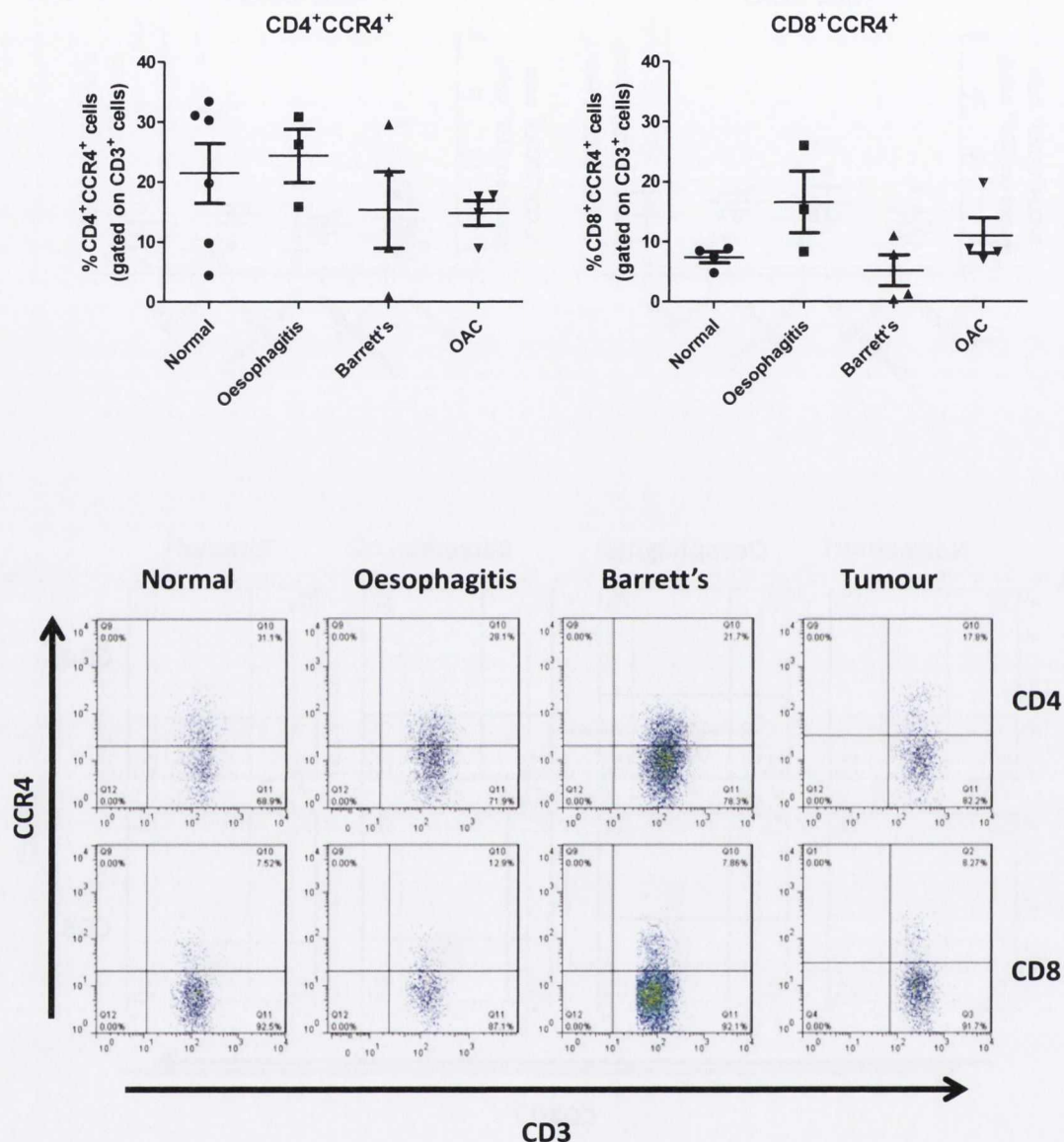


Figure 4.11: CCR4 expression on oesophageal infiltrating T cells.

Oesophageal biopsies were enzymatically digested and immunophenotyped by flow cytometry. Expression of CD3, CD4, CD8 and CCR4 were measured. A) Scatter plot showing percentage of CD4⁺ or CD8⁺ T cells expressing CCR4. B) Flow plots show CCR4 expression on CD4⁺ and CD8⁺ cells within normal, oesophagitis, Barrett's and tumour tissue. All data is presented as mean ± SEM, n=3-6, One way ANOVA, Dunns post-test. N= (Normal =6, Oes=3 BO=4, OAC=4).

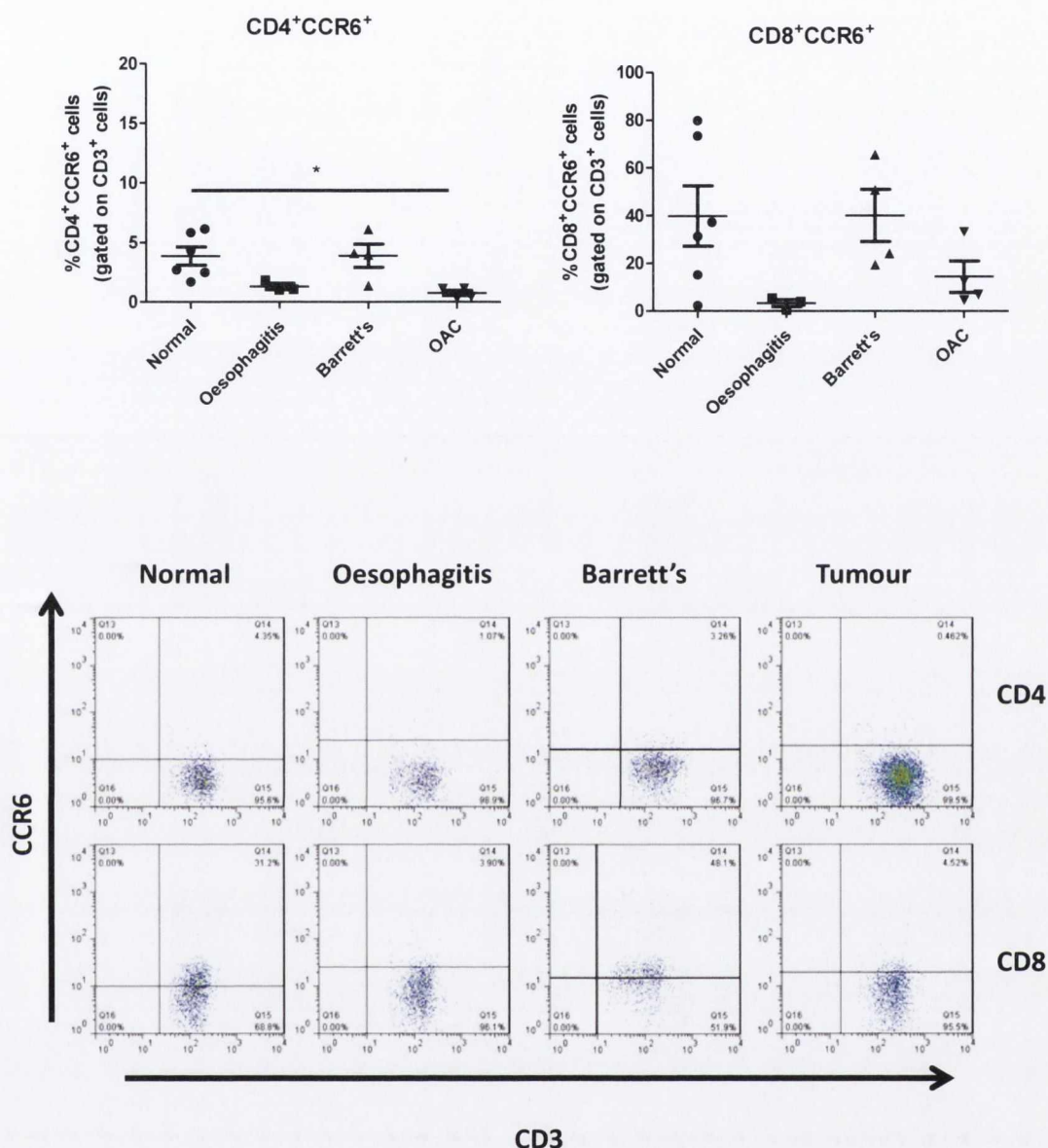


Figure 4.12: CCR6 expression on oesophageal infiltrating T cells.

Oesophageal biopsies were enzymatically digested and immunophenotyped by flow cytometry. Expression of CD3, CD4, CD8 and CCR6 were measured. A) Scatter plot showing percentage of CD4⁺ or CD8⁺ T cells expressing CCR4. B) Flow plots show CCR6 expression on CD4⁺ and CD8⁺ cells within normal, oesophagitis, Barrett's and tumour tissue. All data is presented as mean $\pm$ SEM, n=3-6, *p $\leq$ 0.05, One way ANOVA, Dunns post-test. N= (Normal =6, Oes=3 BO=4, OAC=4).

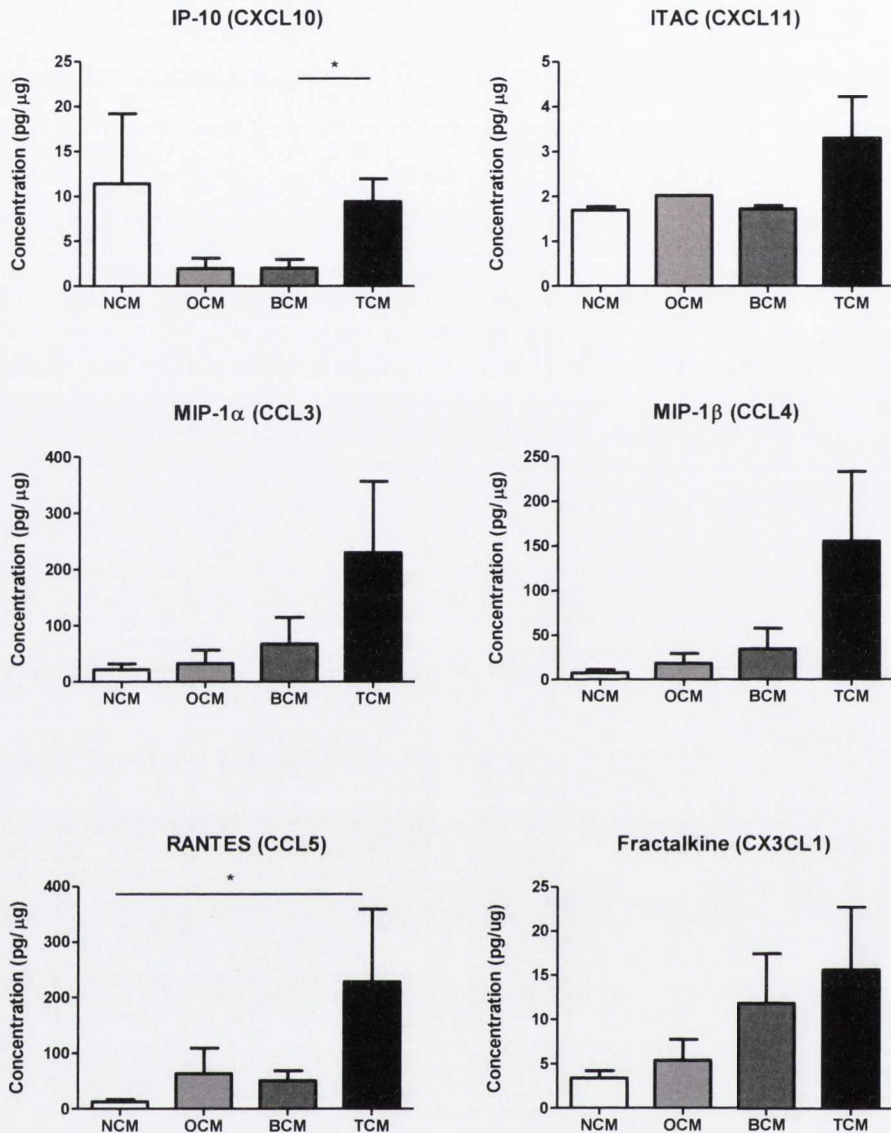


Figure 4.13: Chemokine levels in tissue microenvironment.

Oesophageal biopsies were cultured in X-vivo media for 24hr to generate CM. Chemokine levels (pg/ml) were measured using MSD multiplex and singleplex ELISAs and were normalised to total protein in the biopsy (μg/ml). All data is presented as mean ± SEM, * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, T test (Mann Whitney), n= normal, n=3 oesophagitis, n=7 BO, n=7 OAC.

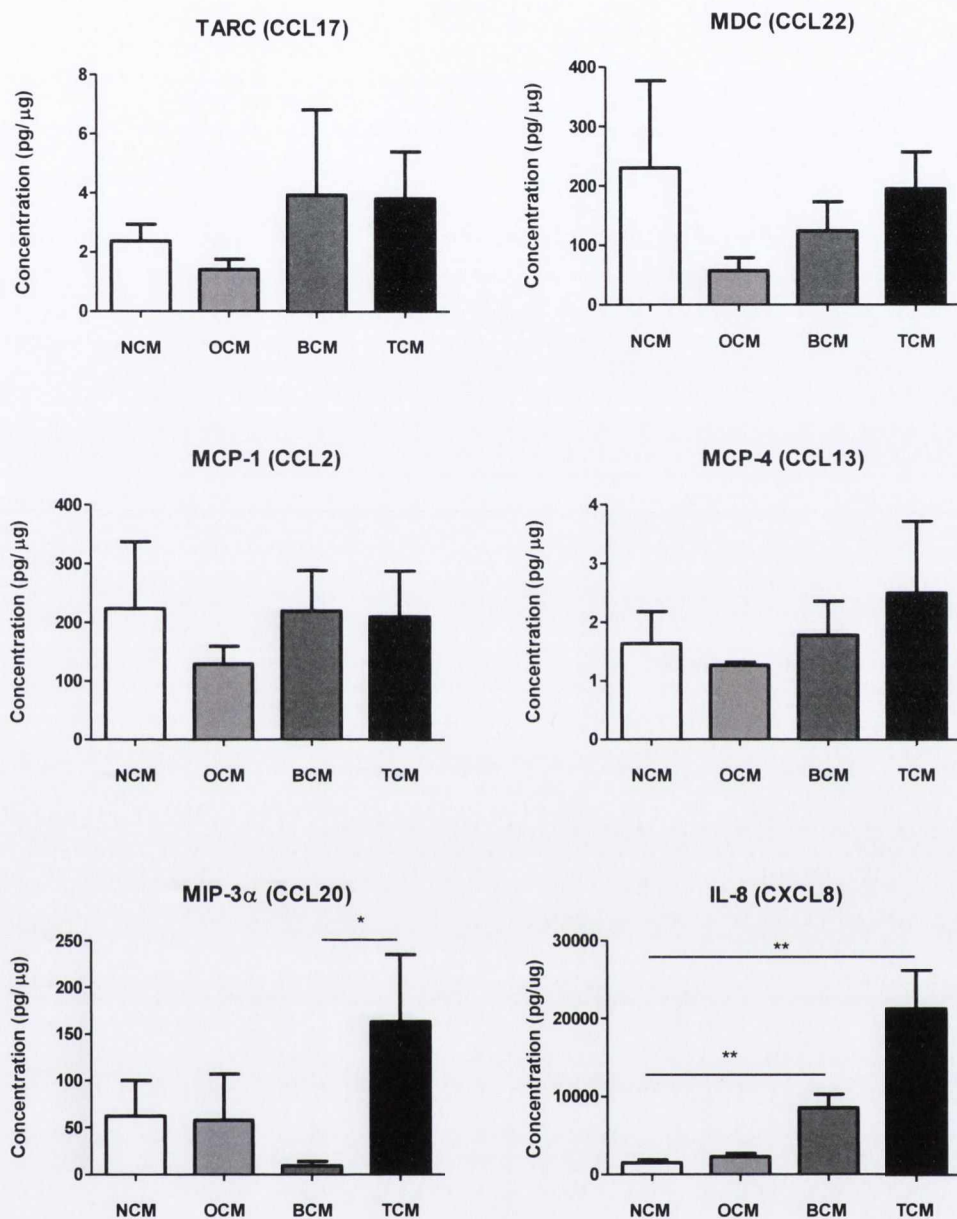


Figure 4.14: Chemokine levels in tissue microenvironment.

Oesophageal biopsies were cultured in X-vivo media for 24hr to generate CM. Chemokine levels (pg/ml) were measured using MSD multiplex and singleplex ELISAs and were normalised to total protein in the biopsy (μg/ml). All data is presented as mean ± SEM, *p ≤ 0.05, ** p < 0.01, *** p < 0.001, T test (Mann Whitney) n= normal, n=3 oesophagitis, n=7 BO, n=7 OAC.

4.2.5 X-VIVO™ 15 serum free media induces lymphocyte chemotaxis

In order to test the hypothesis that secreted chemokines in the local oesophageal microenvironment were responsible for the recruitment of distinct T cell populations during the GORD-BO-OAC sequence, we sought to assess recruitment of T cells to conditioned media. Oesophageal biopsies were cultured in X-VIVO™ media to generate conditioned media (CM); normal (NCM), Barrett's (BCM) and tumour (TCM). X-VIVO™ media provided a serum free media specifically formulated to support T cell growth and proliferation. This allowed us to accurately measure secreted factors from oesophageal tissue, and examine its impact on T cell phenotype and function without contamination from FBS. Migration towards CM was examined using a Boyden chamber assay. Initial results however suggested that X-VIVO™ alone was capable of inducing lymphocyte migration to a similar level to the positive control (20% FBS) (Figure 4.15). X-VIVO™ media increased the number of migrated lymphocytes compared to media only control (1033 ± 449.1 cells vs. 124.2 ± 30.74 cells) similar to the level of lymphocyte recruitment by the positive control (1385 ± 30.74 cells). It is possible that X-VIVO™ media contains factors which are acting as chemoattractants (perhaps in the form of nutrients) for T cells; however the manufacturers would not disclose the exact formulation. For this reason it was unfortunately not possible to determine levels of T cell migration towards oesophageal conditioned media.

4.2.6 The effect of recombinant chemokines in stimulating T cell migration

As it was not possible to determine levels of migration towards oesophageal conditioned media generated, we sought to examine whether T cells from patients across the disease sequence had altered responses to recombinant chemokines identified in our study as being potentially important. PBMC were isolated from the blood of healthy donors, Barrett's and OAC patients, were plated in a transwell plate and allowed to migrate towards the recombinant chemokines CXCL10, CCL3, CCL20, CCL5, CCL17 and CXCL8 (at a concentration of 10ng/ml), or a cocktail of these chemokines (composed of 10ng/ml of each). Recombinant chemokines were used at a concentration of 10ng based on previously optimised studies carried out in the lab and a thorough literature search.

Results are reported as percentage change in number of cells migrated to treatment, compared to number of cells migrated to media only (negative) control. In blood from normal donors, CXCL10 led to an increase in the migration of T cells compared to media only control ($496.3 \pm 325.6\%$ of control) this was somewhat reduced in Barrett's ($227.9 \pm 62.03\%$ of control) and interestingly T cells from OAC patients were unresponsive to Th1 chemokine CXCL10 ($100.1 \pm 30.72\%$ of control)(Figure 4.16). CCL3 led to an increase in T cell migration in cells from normal ($351.6 \pm 185.4\%$ of control) and Barrett's patients ($380 \pm 165.7\%$ of control) but again cells from OAC patients were unresponsive ($127.6 \pm 16.88\%$ of control) (Figure 4.16). CCL20 caused an increase in T cell migration compared to media only control ($446.5 \pm 288.2\%$ of control), which decreased in Barrett's ($225.8 \pm 57.25\%$ of control) and was again lost in OAC patients ($93.26 \pm 20.59\%$ of control). Similar levels of migration towards CCL5 were observed in normal ($519.6 \pm 379.9\%$ of control) and Barrett's patients (582.5 ± 360.4), however, this was again lost in cells from OAC patients ($70.0 \pm 19.54\%$ of control). CCL17 induced T cell migration in cells from normal ($410.5 \pm 214.9\%$ of control) and Barrett's patients ($330.2 \pm 178.5\%$ of control), T cells from OAC patients were again unresponsive ($80.2 \pm 25.28\%$ of control). CXCL8 induced T cell migration in cells from normal patients ($316.5 \pm 135.7\%$ of control), this migration was further increased in cells from Barrett's patients ($634.4 \pm 464.6\%$). Similarly when a cocktail of these chemokines was used, T cell migration increased in cells from normal donors ($596.8 \pm 30.9\%$ of control) and Barrett's patients ($227.9 \pm 29.27\%$ of control) but it was not able to induce migration in cells from OAC patients ($100.4 \pm 10.72\%$ of control) (Figure 4.16). Data obtained demonstrated that T cells from normal donors were highly responsive to all chemokines tested; the responsiveness was somewhat reduced in PBMC from Barrett's blood, and completely lost in PBMC from OAC blood. On further examination, the number of number of T cells migrating to media only control was consistently higher in cells from OAC patients (337.6 ± 151.8 cells) compared to cells from normal (95.45 ± 50.24 cells) and Barrett's (105.6 ± 27.76 cells) (Figure 4.17) suggesting that cells in OAC may be more motile and the response is not specific to chemokines.

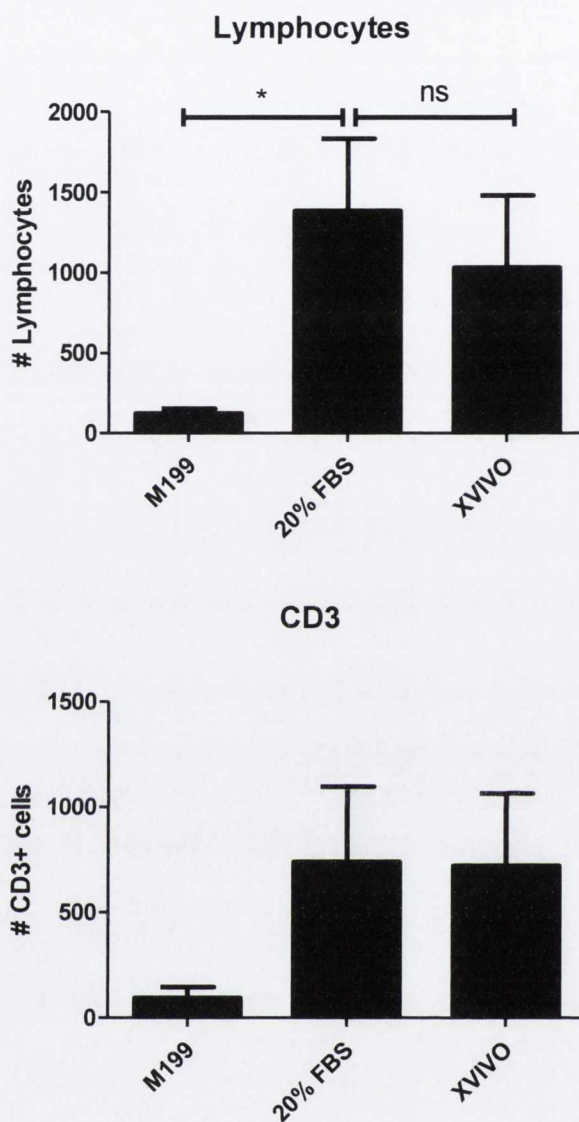


Figure 4.15: Chemotactic effect of X-vivo media.

Healthy PBMC were plated in top chamber of transwell plate. 20% FBS (positive control) or X-vivo media, or M199 media alone (negative control) were placed in the bottom well as a chemoattractant. Bar charts show number of migrated cells, * $p < 0.05$ paired t-test, $n = 3$.

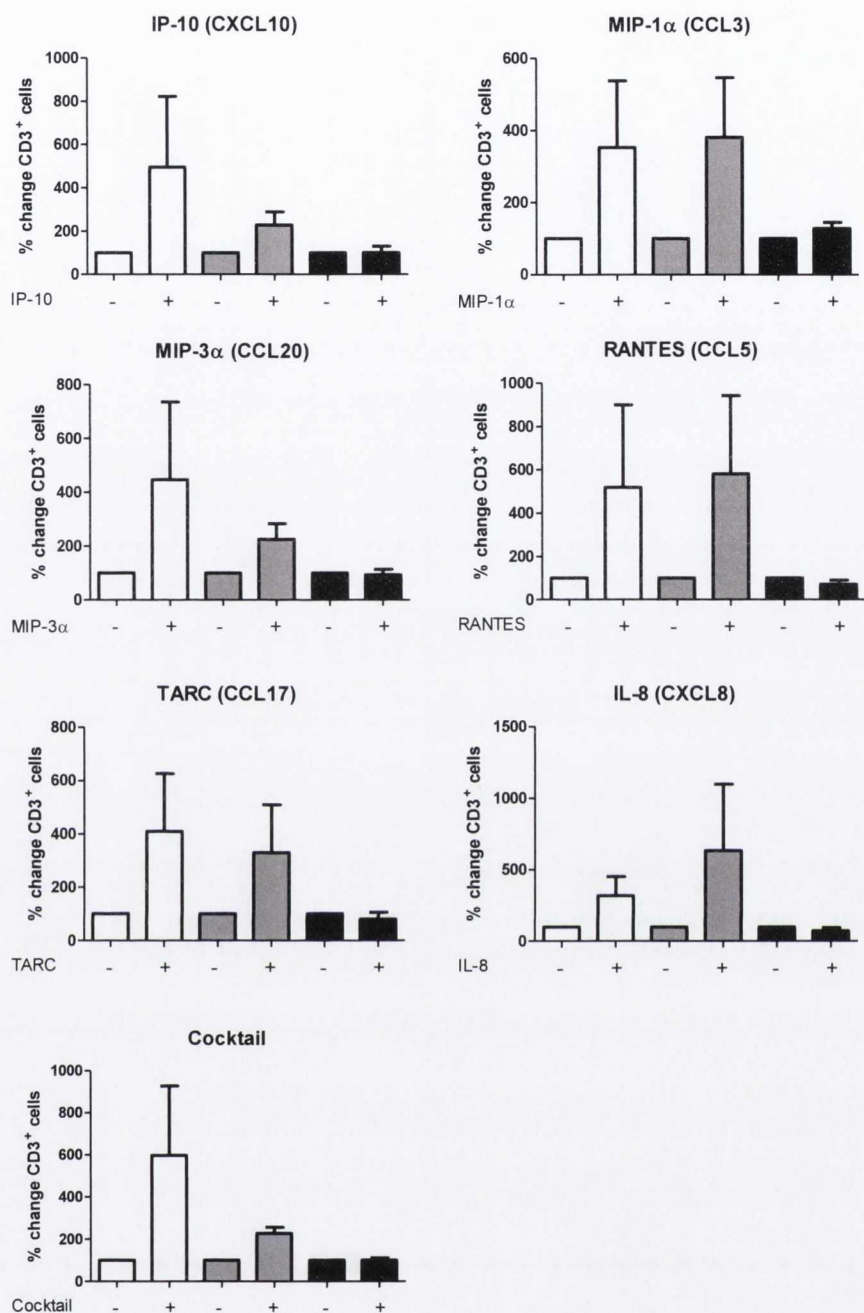


Figure 4.16: Chemotaxis of CD3⁺ T cells to recombinant chemokines (presented as % change).

PBMC from normal donors, Barrett's and OAC patients were plated in top chamber of transwell plate. Recombinant chemokines (10ng/ml) were placed in the bottom chamber, and were incubated for 2hr. Cocktail was composed of 10ng/ml each of CXCL10, MIP-1α, MIP-3α, RNATES, TARC and CXCL8. Bar charts show T cell migration expressed as a percentage of migrated cells to media only control (set to 100%). All data is presented as mean ± SEM, n=3. Paired t test was carried out between treatments (bars of same colour), unpaired t test was carried out between disease stages.

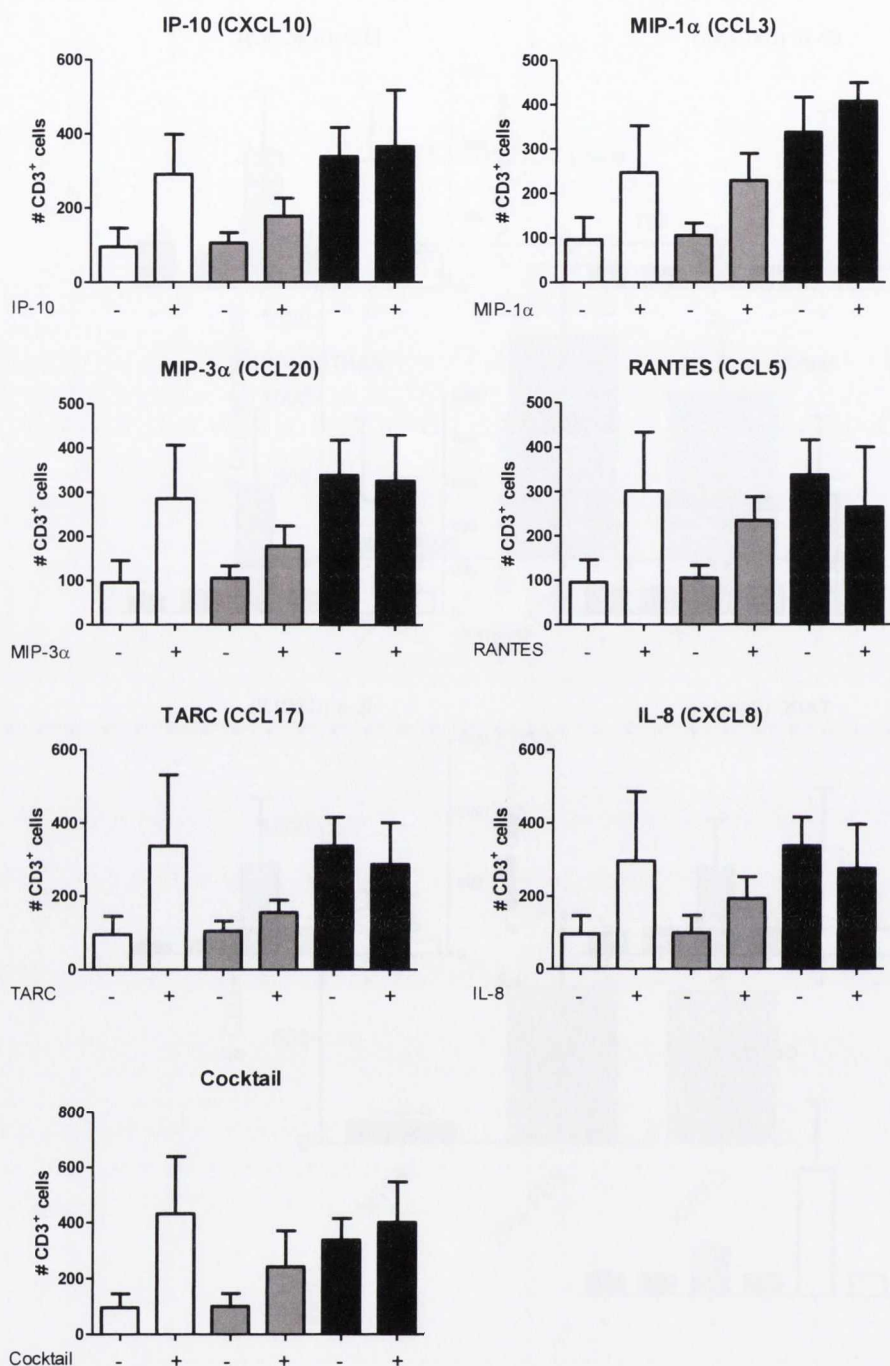


Figure 4.17: Chemotaxis of CD3⁺ T cells to recombinant chemokines.

PBMC from normal donors, Barrett's and OAC patients were plated in top chamber of transwell plate. Recombinant chemokines (10ng/ml) were placed in the bottom chamber, and were incubated for 2hr. Cocktail was composed of 10ng/ml each of CXCL10, MIP-1α, MIP-3α, TARC and CXCL8. Bar charts show number of migrated cells. All data is presented as mean ± SEM, n=3. Paired t test was carried out between treatments (bars of same colour), unpaired t test was carried out between disease stages.

4.2.7 Analysis of chemokine receptor blockade on T cell chemotaxis

To examine whether chemokine receptor blockade could represent a viable therapeutic option to alter T cell accumulation within diseased oesophageal tissue, PBMC were pre-treated for 1hr with antagonists to CXCR3, CXCR1, CCR3, CCR4 and CCR5, and effect on T cell migration towards the chemokine cocktail was examined. Interestingly, cells from Barrett's patients appeared to be the most responsive to receptor blockade, while cells from normal and OAC patients showed little effect.

In cells from healthy donors, pre-treatment with receptor antagonists CXCR3 (AMG487), CXCR1 (SCH527123), CCR3 (SB328437) and CCR4 (C021 dihydrochloride) had no effect on chemotaxis towards chemokine cocktail (Figure 4.18). The CCR5 antagonist (Vicriviros Malate) was the only antagonist identified to reduce T cell chemotaxis towards the chemokine cocktail (432.6 ± 205.6 cells to 173.1 ± 142.7 cells). In PBMC from Barrett's patients, pre-treatment with the CXCR3 antagonist reduced T cell migration towards chemokine cocktail back to baseline levels (243.0 ± 128.0 cells to 80.79 ± 23.33 cells). Similar finding were observed with the CXCR1 antagonist (243.0 ± 128.0 cells to 114.5 ± 51.8 cells), CCR3 antagonist (243.0 ± 128.0 cells to 150.8 ± 79.1 cells), CCR4 antagonist (243.0 ± 128.0 cells to 82.43 ± 29.19 cells). Pre-treatment with the CCR5 antagonist did not alter T cell migration towards chemokine cocktail (Figure 4.18). T cells obtained from OAC patient blood did not demonstrate any response to receptor blockade. These results suggest that cells from Barrett's patients are the most amenable to receptor blockade, and this may be a viable therapeutic option to prevent Th2 cell migration to Barrett's tissue; whereas targeting chemokine receptors in OAC is unlikely to have any response.

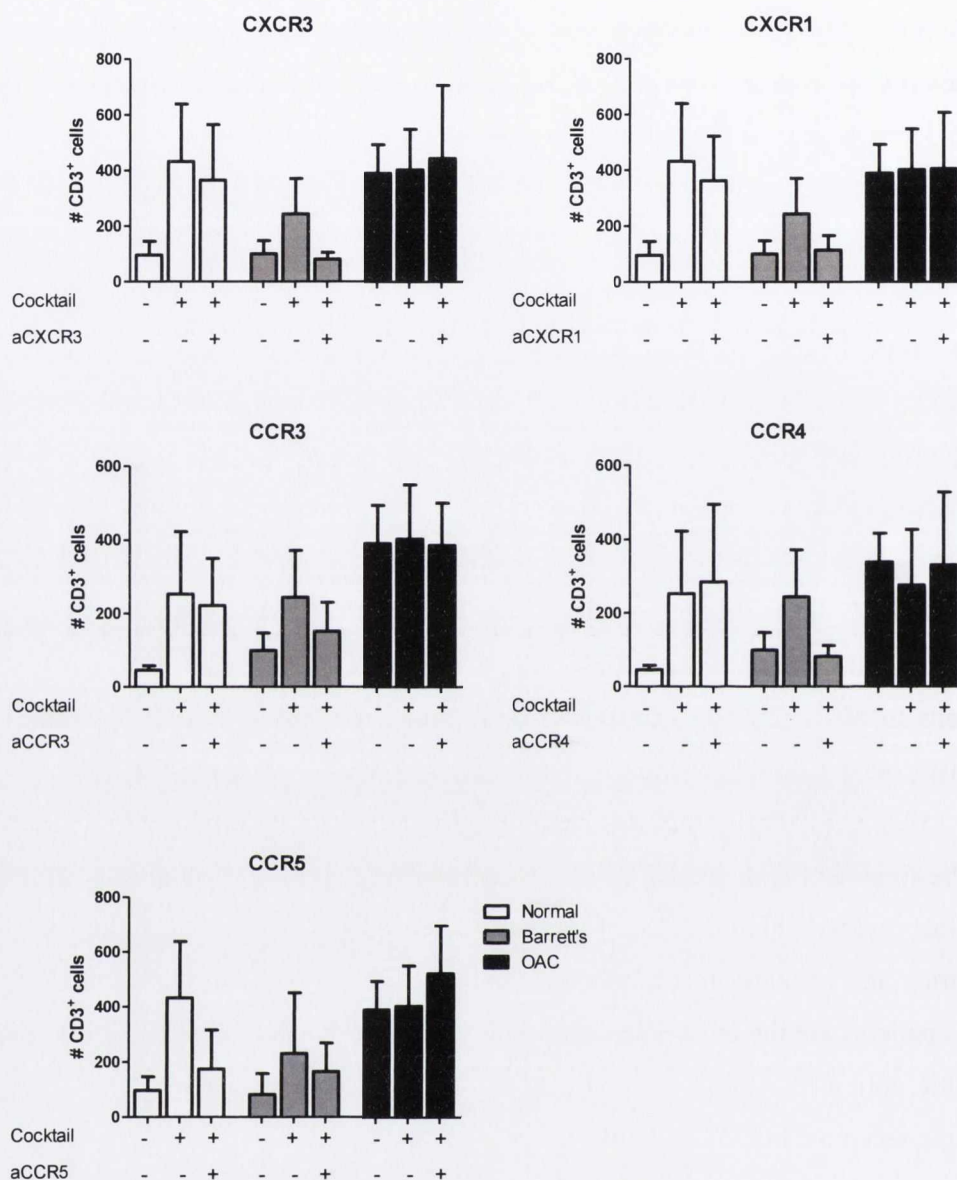


Figure 4.18: Blockade of chemokine receptors.

PBMC from normal donors, Barrett's and OAC patients were pre-treated with chemokine receptor antagonists for 1hr prior to chemotaxis assay. Cells were plated in top chamber of transwell plate and a cocktail of recombinant chemokines (composed of 10ng/ml each of CXCL10, MIP-1 α , MIP-3 α , RNATES, TARC and CXCL8) were placed in the bottom chamber and allowed to migrate for 2hr. Bar charts show T cell migration expressed as a percentage of migrated cells to media only control (set to 100%). All data is presented as mean $\pm$ SEM, n=3. Paired t test was carried out between treatments (bars of same colour), unpaired t test was carried out between disease stages.

4.3 Discussion

While chemokines are now accepted to play a role in the initiation and maintenance of inflammation in GORD, the chemokine profile in the oesophagitis-metaplasia-carcinoma sequence has not yet been assessed. Previous studies have suggested inflammatory gradients in oesophageal tissue as it progresses through GORD, BO and OAC [106, 107, 235]. As we and others have described, GORD is characterised by a pro-inflammatory or Th1 response, BO has been reported as a more humoral type response with increased levels of Th2 cells and cytokines, while in OAC we have reported a mixed inflammatory profile. Increased levels of CCL5, CXCL8 and CCL2 have been described in oesophagitis tissue and are thought to result in the recruitment of immune cells to oesophageal tissue. The mechanism for the accumulation of Th2 type cells in BO has however, not yet been described. In the previous chapter we observed minimal T cell proliferation as a result of secreted factors in the Barrett's microenvironment; thus the hypothesis of this chapter was that the production of chemokines in the local oesophageal microenvironment may contribute to the distinct T cell recruitment. This effect can be seen in other conditions including Hodgkin's lymphoma, in which the malignant reed Sternberg cells express a range of Th2 chemokines causing the local suppression of Th1 cellular immune response [184]. Another example is in Kaposi's sarcoma in which the Kaposi sarcoma associated herpes virus encodes the chemokines vMIP1 (viral macrophage inflammatory protein), vMIP2 and vMIP3, which selectively recruit polarized Th2 cells [259] thus, establishing a tumour promoting environment.

CXCR3 and CCR5 pathways and Th1 cells

CCR5 and CXCR3 are the predominant receptors expressed on Th1 cells. CXCR3 binds the chemokines CXCL9, CXCL10 and CXCL11 to induce the migration of activated T cells [172-174]. CCR5 is a receptor for the inflammatory chemokines CCL5, CCL3 and CCL4 and is also found on activated Th1 cells [171]. Our data showed higher levels of CXCR3⁺ cells both in circulation and within the oesophageal tissue of oesophagitis patients. Previous studies have identified a role for CXCR3 in promoting T cell infiltration and an effective anti-tumour immune response in melanoma patients, with studies demonstrating an association between CXCR3 expression by tumour specific CD8⁺ T cells and patient survival [260]. Furthermore, increased expression of the ligands CXCL10 and CXCL9 has been shown to promote

T cell infiltration and may therefore promote an effective anti-tumour immune response [261, 262]. We therefore hypothesised that the increase in CXCR3⁺ in oesophagitis tissue may be due to higher levels of its chemokines within the oesophageal microenvironment, however levels of CXCL10 and CXCL11 were found at lower levels in the OCM, suggesting that they do not play a key role in the recruitment of these cells early in disease. We previously demonstrated that factors in the OCM can significantly enhance T cell proliferation; therefore it is possible that during oesophagitis the expansion of resident Th1 cells in the oesophageal results in the characteristic T cell profile and T cell recruitment does not play a vital role.

Increased proportions of CCR5⁺ cells were observed within Barrett's tissue compared to normal. To the best of our knowledge this is the first time this has been described in BO, and may represent a pathway through which Th1 cells could be recruited to BO tissue. Indeed CCL3, CCL4 and CCL5 showed a slight increase, although not significant, in the BCM. Given that BO is characterised by a Th2 profile and we have previously demonstrated increased proportions of IL-10 and IL-4 producing cells in BO tissue, the increase in CCR5⁺ cell was unexpected. It is known in the setting of BO that reflux oesophagitis can co-exist simultaneously. Therefore this increase in CCR5⁺ cells may represent the presence of reflux oesophagitis in this cohort.

OAC patients displayed significantly reduced proportions of CXCR3⁺ T cells. We hypothesised that this may represent the recruitment of CXCR3⁺ cells to tumour tissue as results demonstrated increased levels of CXCL10 and CXCL11 in TCM. Indeed previous studies have identified higher levels of CXCL10 in oesophageal cancer patients [263], and have demonstrated its role in T cell recruitment to tumour tissue [261, 262]. However, no difference was observed in the proportion of CXCR3⁺ or CCR5⁺ cells within tumour tissue. This could possibly suggest that this pathway is not at play in the recruitment of Th1 cells to OAC tissue. Depending on the chemokine concentration gradient between serum and tissue microenvironment, CXCR3⁺ or CCR5⁺ may remain in circulation. Indeed levels of CXCL10 were found at higher concentrations within the serum of OAC patients compared to the TCM, however a direct comparison cannot be made here as TCM was generated using an *ex vivo* model and thus is not representative of the absolute levels in the tissue. Alternatively, chemokine receptors are known to be internalised following ligand binding. A study by

Meiser *et al.*, demonstrated that binding of CXCR3 to CXCL11 could induce rapid receptor internalisation followed by proteasomal degradation [264], with similar effects seen following CXCL10 and MIG binding [265]. As such the reduction in CXCR3⁺ cell in OAC tissue could represent their recruitment and subsequent internalisation of the receptors. Thus further work is needed to clarify whether this pathway is responsible for the recruitment of Th1 cells to OAC tissue.

CCR4/ CCR3 pathways and Th2 cells

Th2 cells primarily express CCR3 and, following activation express CCR4 [16, 17]. CCR4 binds to the chemokines CCL17, and CCL22 [171]. CCR3 binds CCL5 and CCL13, amongst others. Since CCR3⁺ cells are increased in Barrett's and OAC blood, this may allow for the recruitment of Th2 cells to oesophageal tissue towards the higher levels of CCL5 observed in TCM. Interestingly, a study by Zhang *et al.*, demonstrated that CCR3/ CCL5 signalling was vital for Th2 polarisation of CD4⁺ T cells [266]. However no change was observed in the levels of CCR3⁺ or CCR4⁺ cells in Barrett's or tumour tissue. This again suggests that either this pathway does not play a role in Th2, or that receptor internalisation has occurred.

CCR6 pathways and Th17 cells

CCR6 is the only known receptor for CCL20 and is found to be expressed on Th17 cells, memory T cells and dendritic cells [171]. Our data demonstrated lower levels of circulating CCR6⁺ T cells in OAC patients. While serum levels of CCL20 were shown to be low in OAC patients, levels were higher in TCM providing a mechanism whereby CCR6/CCL20 may recruit Th17 cells to the tumour tissue. However within the tumour tissue our data did not support this theory as decreased levels of CCR6⁺ cells were observed. Furthermore in chapter 3 we demonstrated significantly reduced proportions of IL17 producing CD4⁺ T cells in OAC tissue. Thus our results suggest that the CCR6/CCL20 pathway does not induce Th17 T cell recruitment to OAC tissue. Previous work by Chen *et al.*, identified increased levels of IL-17 producing T cells in the blood and tumour tissue of oesophageal cancer patients [96], however this study used a mix of OAC and squamous cell carcinoma and so cannot be directly compared to our results.

Immunotherapeutic targeting

While this study carried out a detailed immunophenotyping of chemokine receptor expression on T cells throughout the GORD-BO-OAC sequence, the overall aim was to identify possible immunotherapeutic targets to prevent the recruitment of tumour promoting Th2 cells to BO tissue thus halting disease progression. T cells from normal donors proved equally responsive to all chemokines tested, however, T cells from BO patients demonstrated preferential migration towards the Th2 chemokine CCL17, as well as CCL3, CCL5, and CXCL8 compared to the Th1 chemokine CXCL10 or to MIP-3 α . This suggests that these chemokines, specifically CCL17, may be key therapeutic targets in altering T cell trafficking to oesophageal tissue.

Interestingly receptor blockade in cells from normal patients showed little efficacy, with only the CCR5 antagonist showing any reduction in T cell migration. However cells from Barrett's patients proved far more amenable to receptor blockade. As BO cells appeared to have preferential migration towards CCL3, CCL5 and CCL17, this study suggests a role for CCR3 and CCR4 in recruitment of Th2 cells to BO tissue. Thus inhibition of CCR3 or CCR4 signalling may be a viable therapeutic target. In accordance with this, our results demonstrated that CCR3 and CCR4 receptor blockade could block T cell migration in BO blood. CCR4 and its ligands CCL17 and CCL22, have been suggested to play a role in the pathogenesis of atopic dermatitis, allergic asthma, gastric and renal cell carcinoma [267-271]. However, their role in the oesophagitis-BO-OAC sequence has yet to be examined. A number of studies by Nakagami *et al.*, have demonstrated the potential of CCR4 antagonism in experimental models of allergic diseases, including asthma and atopic dermatitis. They demonstrated that CCR4 antagonism could significantly inhibit CCL17 induced Th2 T cell migration, ovalbumin induced airway inflammation [272], and ovalbumin induced ear swelling while reducing serum levels of IL-4 [273]. CCR4 is not only expressed by Th2 cells, it is also found on the surface of regulatory T cells. Thus targeting the CCR4 pathway may also inhibit Treg recruitment and potentially enhance antitumour activity. In 2012, a humanised CCR4 mAb - Mogamulizumab was approved for the treatment of adult T cell leukaemia (ATL) in Japan, and is currently in clinical trials in the USA and Europe for the treatment of ATL and peripheral T cell lymphoma [274, 275]. In phase II clinical trials Mogamulizumab, resulted in a lasting decrease in CD4⁺CCR4⁺ cells and regulatory T cells, demonstrated anti-tumour activity [275], and has shown enhanced

antibody dependant cellular cytotoxicity (ADCC) against primary tumour cells from ATL patients both in vitro and in a humanised mouse model [257, 258, 276]. Interestingly, PBMC from OAC patients displayed lower responsiveness to chemokines and proved less amenable to receptor blockade. This indicates that chemokine receptor blockade may be a promising target at the Barrett's stage of disease progression but not at the OAC stage where multiple pathways are already at play with a mixed Th1/Th2 profile observed.

For the first time this study characterised the chemokine/chemokine receptor profile in the GORD-BO-OAC sequence. We identified a number of pathways that may potentially play a role in T cell recruitment to oesophageal tissue. While in chapter 3 we suggest that immune cell profile in oesophagitis is mediated by the increased proliferation of resident T cells; in BO we suggest that the Th2 profile observed is potentially mediated by the active recruitment of T cells to oesophageal tissue. In addition we demonstrated that during the BO stage of disease progression, T cells remain amenable to chemokine receptor blockade and suggest that blocking the recruitment of CCR4⁺ and CCR3⁺ cells could dampen down the Th2 response in BO and potentially prevent the progression to OAC.

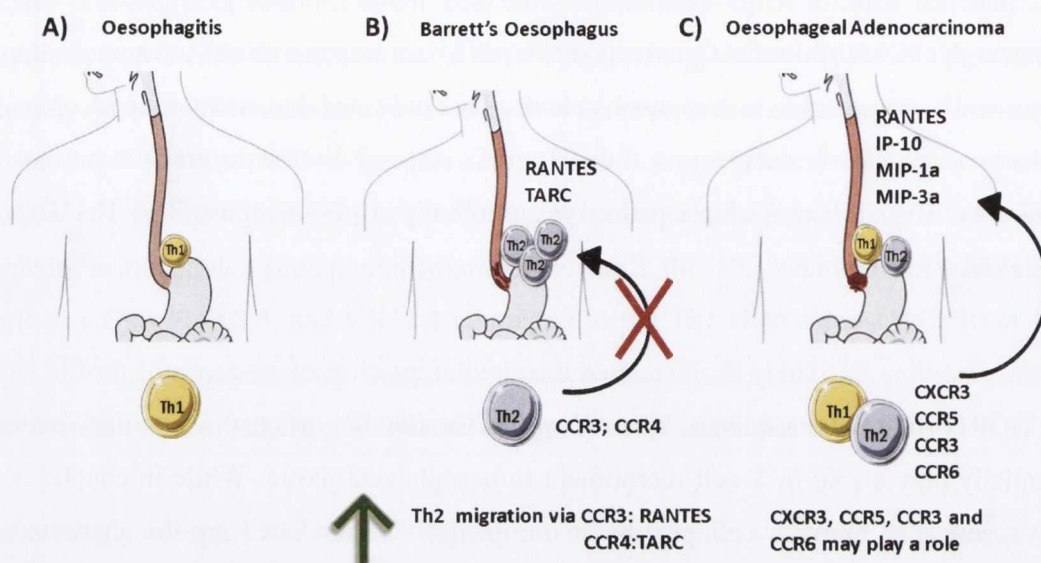


Figure 4.19: Summary of findings from chapter 4.

A) Results from this chapter did not identify a role for chemokines in the recruitment of T cells to oesophagitis tissue. B) Results identified a potential role for the CCR3 and CCR4 pathways in the recruitment of Th2 cells to Barrett's tissue towards increased levels of and TARC within the BO microenvironment. Blocking the CCR3 and CCR4 receptors resulted in reduced T cell migration suggesting a potential immunotherapeutic target. C) Results identified increased levels of , CXCL10, MIP1 α and MIP-1 β within the OAC microenvironment. However circulating T cells from OAC patients were not found to respond to these chemokines, indicating altered migratory pathways at this disease stage.

5. Chapter 5- Results

Identification of metabolic challenges faced by T cells in BO and OAC tissue and assessing the impact on T cell profile.

5.1 Introduction

It is well known that tumour cells upregulate glycolysis even in the presence of adequate oxygen [194]. This highly glycolytic phenotype has been demonstrated in numerous cancer types including breast, gastric, oesophageal squamous and lung carcinoma [196], with studies from our own department demonstrating increased rates of both glycolysis and oxidative phosphorylation in Barrett's and oesophageal adenocarcinoma tissue [37]. This results in the increased uptake of glucose from the local microenvironment and can therefore create a nutrient poor and harsh environment for infiltrating and resident T cells. This metabolic pressure within the oesophageal tissue may significantly impact T cell metabolism and function, and thus understanding metabolic changes in the GORD-BO-OAC sequence may identify potential immunotherapeutic targets.

While naïve T cells primarily rely on oxidative phosphorylation (OxPhos) as their primary source of energy [197-199], upon antigen recognition and activation, they must upregulate glycolysis to fuel the rapid cell growth and proliferation and to allow for the acquisition of effector functions such as cytokine production [197]. While oxidative phosphorylation is a highly efficient process in terms of ATP generation, generating 36 molecules of ATP per molecule of glucose [277]; glycolysis produces only 2 molecules of ATP [186]. However, glycolysis supplies the cell with metabolic intermediates for lipid, protein and carbohydrate synthesis [186], thus providing distinct advantages for rapidly dividing cells. While effector T cells are relatively short lived, memory T cells have a far longer life span and therefore have different metabolic requirements. Similar to naïve T cells they rely on OxPhos [197, 201, 202]. Effector T cells are extremely dependent on glucose availability, and failure to upregulate glycolysis in the absence of glucose can result in decreased proliferation, reduced cytokine expression and T cell anergy [203, 204]. Regulatory T cells however, are far less dependent on glucose and primarily rely on lipid oxidation [205, 206]. These differences in T cell metabolism mean that individual T cell subsets will respond differently to metabolic challenges in the local environment. Thus limited glucose availability in the tumour microenvironment may selectively inhibit effector T cells while providing a distinct survival advantage to regulatory T cells.

The upregulation of glycolysis is critical for T cell activation and therefore limited glucose availability in the tumour microenvironment may lead impaired effector function and immunosuppression. Indeed studies have demonstrated that glucose deprivation can inhibit T cell proliferation and IFN- γ production [278, 279] and induce T cells to enter a state of functional inactivity or anergy [280]. The increased glycolytic rate of tumour cells imposes further challenges upon infiltrating and resident immune cells. It can result in the acidification of the extracellular environment through increased H⁺ and lactate production during glycolysis [223, 224]. Thus tumour cells can have an extracellular pH of between 6 and 6.5 [225-227] and can significantly impact immune cell function. In human and mouse specific CD8⁺ T cells, a pH of 6-6.5 can induce an anergic state characterised by impaired cytolytic activity, IL2R expression and cytokine secretion [229]. Thus understanding the metabolic challenges faced by T cells in the oesophageal microenvironment may identify potential targets to alter T cell metabolism and enhance an anti-tumour immune response.

In chapter 3 we demonstrated reduced levels of activated T cells within OAC tissue, and hypothesise that this may represent the inability of TILs to upregulate glycolysis due to reduced nutrient availability. The enhanced metabolic profile in Barrett's and tumour tissue could potentially produce an immunosuppressive environment for T cells, driven by the reduced availability of glucose and acidic pH in the local oesophageal microenvironment. The overall aims of this chapter were as follows:

- To examine metabolic profile of T cells within normal, Barrett's and OAC tissue
- To measure levels of glucose, lactate and other metabolites in the oesophageal microenvironment
- To determine the impact of glucose deprivation on T cell metabolism, activation and cytokine expression
- To determine the impact of low pH on T cell metabolism, activation and cytokine expression
- To inhibit metabolic pathways to overcome T cell immunosuppression and alter T cell phenotype

5.2 Results

5.2.1 Tumour infiltrating lymphocytes display reduced glycolysis

To assess the metabolic profile of T cells infiltrating oesophageal tissue throughout the Barrett's to adenocarcinoma sequence, oesophageal biopsies from normal, Barrett's and OAC tissue were enzymatically digested and stained for markers of glycolysis (GAPDH and GLUT1) and oxidative phosphorylation (HSP60 and ATP5B). Characterisation was carried out on CD3⁺ population. Results demonstrated a significant reduction in the mean fluorescent intensity of GAPDH in tumour infiltrating T cells compared to normal (4.08 ± 0.77 vs. 13.50 ± 1.67 , $p < 0.05$). Intensity of GLUT1, HSP60 and ATP5B remained unaltered across the sequence (Figure 5.1). This suggests that glycolysis may be reduced in OAC infiltrating T cells.

5.2.2 NMR analysis of metabolite levels in Barrett's microenvironment

In order to identify the major metabolic pathways at play during disease progression, key metabolites in the oesophageal microenvironment were examined by NMR spectroscopy for this study by Dr. Lorraine Brennan, Institute of Food and Health, Conway Institute, UCD. The largest components of the conditioned media were lactate and glucose. Glucose accounted for between 27 and 38% of metabolites identified, lactate accounted for 30 and 38% of metabolites (Figure 5.2). A significant decrease in the percentage of glucose was observed in BCM ($33.0 \pm 0.43\%$, $p = 0.01$) and TCM ($33.29 \pm 2.41\%$, $p = 0.03$) compared to NCM ($37.26 \pm 0.43\%$), alongside a slight increase in lactate in BCM ($34.41 \pm 1.34\%$, $p = 0.06$) and TCM ($35.50 \pm 3.17\%$, $p = 0.05$) compared to NCM ($31.82 \pm 0.45\%$). Citrate, an intermediate of the TCA cycle, was increased in the BCM ($0.48 \pm 0.047\%$, $p = 0.03$) and TCM ($0.5 \pm 0.08\%$, ns $p = 0.07$) compared to NCM ($0.27 \pm 0.04\%$, $p = 0.03$) (Figure 5.2). These results suggest an increase in both glycolysis and oxidative phosphorylation within Barrett's and OAC tissue compared to normal. Other metabolites identified were acetate, alanine, creatinine, isoleucine, leucine, proline, pyroglutamate and valine; however no significant differences were observed in these metabolites (Figure 5.3).

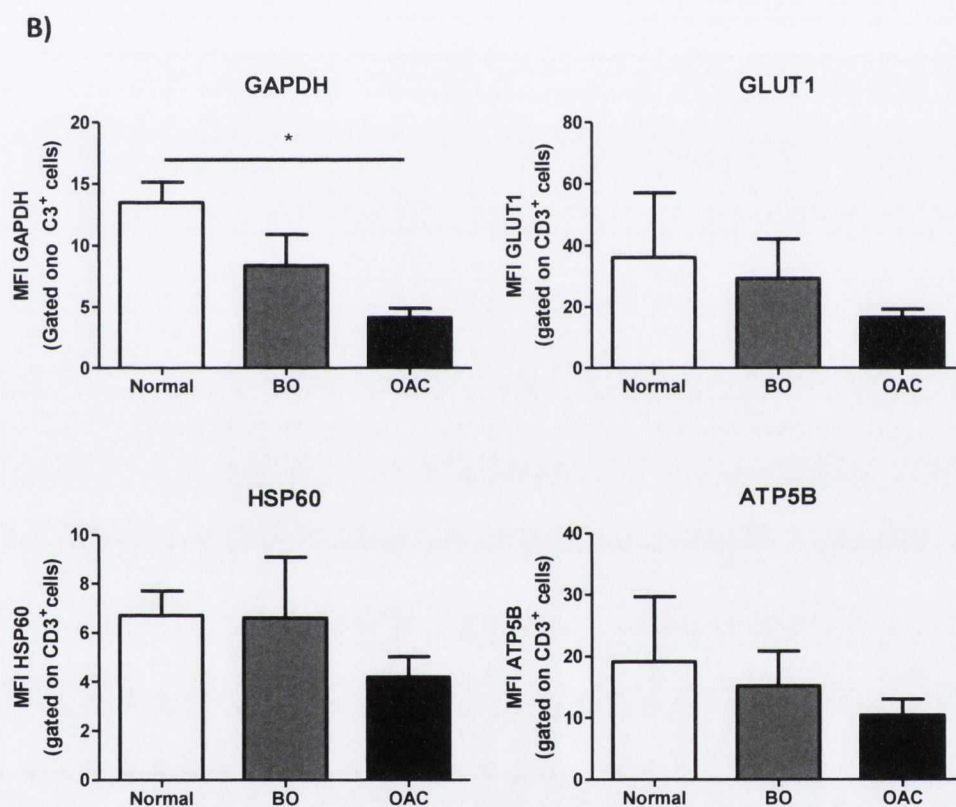
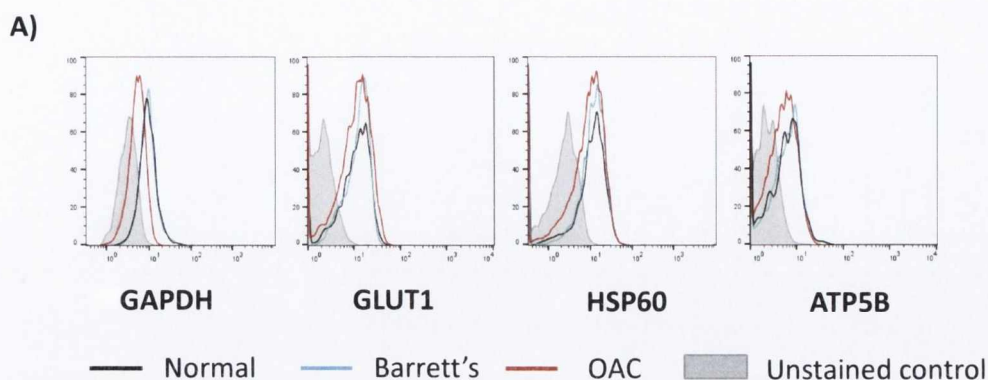


Figure 5.1: OAC infiltrating T cells display decreased glycolysis.

Oesophageal biopsies were enzymatically digested and immunophenotyped by flow cytometry. Expression of CD3, CD8, GAPDH, GLUT1, HSP60 and ATP5B were measured. **A)** Representative histograms showing mean fluorescent intensity. **B)** Bar charts showing percentage of CD3⁺ T cells expressing GAPDH, GLUT1, HSP60 or ATP5B. All data is presented as mean $\pm$ SEM, * $p \leq 0.05$, unpaired t test, $n=3-4$ for all groups.

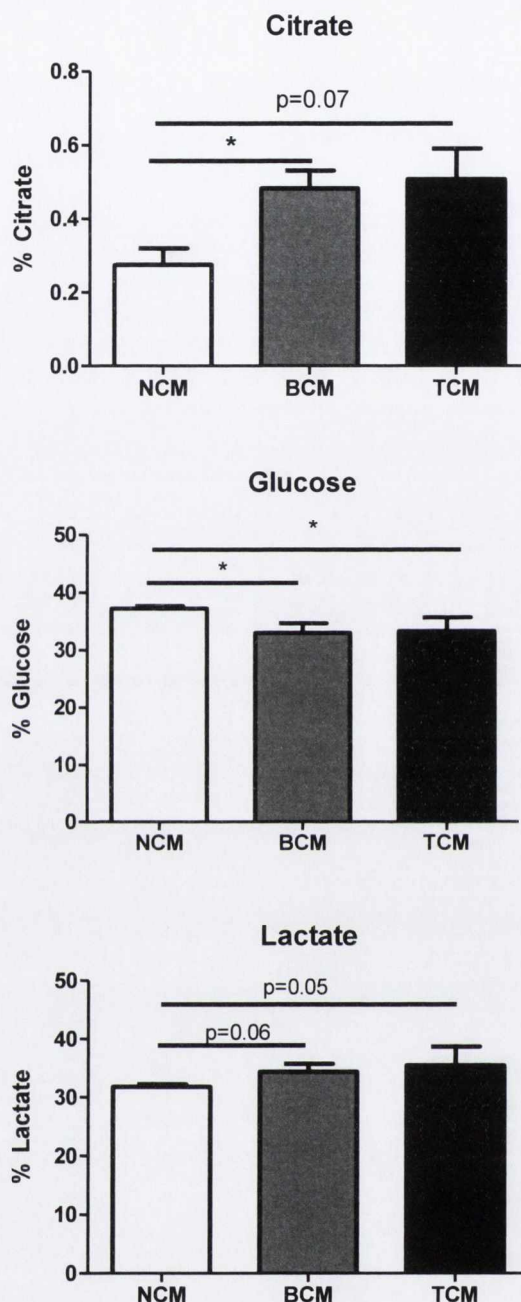


Figure 5.2: Metabolite levels in oesophageal tissue microenvironment indicate increased glycolysis and oxidative phosphorylation.

Oesophageal biopsies were cultured in X-vivo media for 24hr to generate oesophageal conditioned media (CM) (NCM=Normal, OCM=Oesophagitis, BCM= Barrett's, TCM= Tumour). The most prominent metabolites were identified by NMR and were expressed as a percentage of CM. Bar charts show percentage citrate, lactate and glucose in CM, * $p \leq 0.05$, t test (Man Whitney), $n=3-5$.

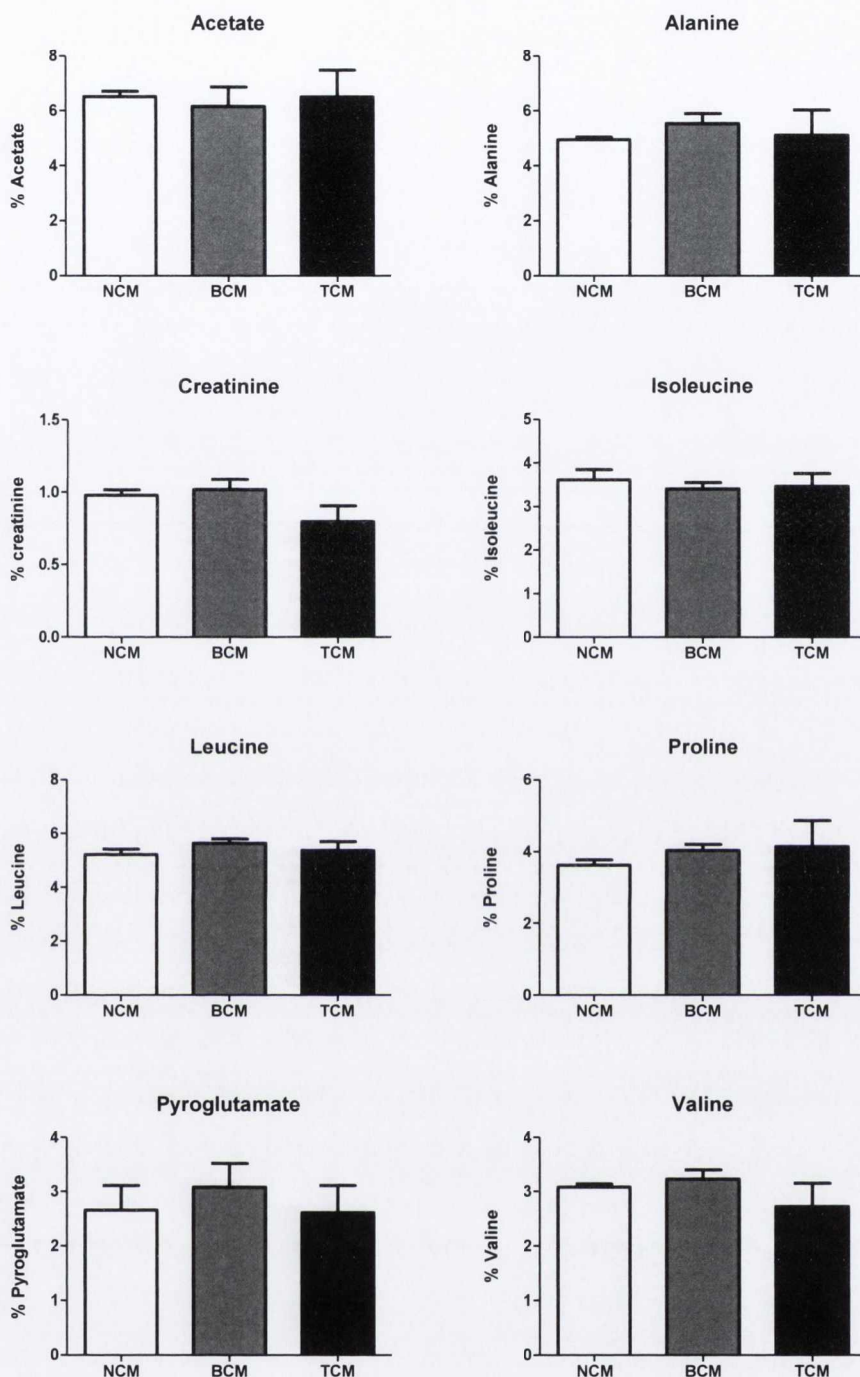


Figure 5.3: Metabolite levels in oesophageal tissue microenvironment.

Oesophageal biopsies were cultured in X-vivo media for 24hr to generate oesophageal conditioned media (CM) (NCM=Normal, OCM=Oesophagitis, BCM= Barrett's, TCM= Tumour). The most prominent metabolites were identified by NMR and were expressed as a percentage of CM. Bar charts show percentage of acetate, alanine, creatinine, isoleucine, leucine, proline, pyroglutamate and valine in CM $\pm$ SEM, t test (Mann Whitney), n=3-5.

5.2.3 Tumour tissue displays increased glycolysis

To further analyse this change in metabolism and to validate the NMR results we sought to examine levels of glucose and lactate across the disease sequence. Data demonstrated a significant decrease in glucose levels in TCM ($2303 \pm 630\text{ng}/\mu\text{g}$) compared to NCM ($4749 \pm 613.5\text{ng}/\mu\text{g}$, $p < 0.05$) and a significant increase in lactate in TCM ($345.3 \pm 92.8\text{ng}/\mu\text{g}$) compared to NCM ($123.8 \pm 14.85\text{ng}/\mu\text{g}$) (Figure 5.4). This data again suggests increased glycolysis in tumour tissue.

5.2.4 Effect of low glucose on T cell activation, cytokine expression and metabolism

As we have shown increased glycolysis in the tumour tissue, this would suggest a reduced nutrient availability for other resident or infiltrating immune cells in the microenvironment. We therefore sought to examine the effect of decreased glucose on T cell metabolism, activation profile and cytokine expression. Healthy PBMC were incubated in RPMI with or without glucose and were immunophenotyped by flow cytometry. Low glucose had no significant impact on the expression of CD45RO, CD45RA, CD69 or CD62L (Figure 5.5). However, data indicated a significant increase in the proportion of CD8⁺ T cells expressing IL-10 when cultured in glucose free media compared to control ($0.14 \pm 0.03\%$ vs 0.07 ± 0.01 , $p = 0.03$) (Figure 5.6). This suggests the emergence of a Th2 or Treg population. A significant increase in the proportion of CD4⁺ and CD8⁺ cells expressing IL-4 was observed upon treatment with glucose free media compared to control (CD4: $0.76 \pm 0.08\%$ vs 0.41 ± 0.06 , $p = 0.03$; CD8: $0.83 \pm 0.18\%$ vs 0.42 ± 0.07 , $p = 0.03$), again suggesting the emergence of a Th2 population. No changes were observed in IFN- γ or TNF- α . Low glucose treatment was found to increase ATP5B expression in CD4⁺ and CD8⁺ cells compared to control (CD4: 198.6 ± 68.6 vs 140.5 ± 44.43 , $p = 0.07$; CD8: 201.5 ± 66.71 vs 148.4 ± 46.39 , $p = 0.08$), although this did not reach significance (Figure 5.7). No differences were observed in GLUT1 or GAPDH expression. These results suggest that limited glucose in the microenvironment can drive oxidative phosphorylation and increase the production of Th2 cytokines.

5.2.5 Effect of low pH on T cell activation, cytokine expression and metabolism

As a result of increased glycolysis and increased lactate, the tumour microenvironment is likely to be at a lower pH. Studies have demonstrated that extracellular pH of solid

tumour is around 6.5 compared with the more neutral pH of normal tissues (pH 7.2-7.4) [225-227]. To test the effects of low pH on T cell phenotype, healthy PBMC were incubated for 24hr in RPMI at normal pH (7.0) or low pH (6.5). Incubation of T cells in low pH conditions had no effect on the percentage of cells expressing CD45RO, CD45RA, CD69 or CD62L (Figure 5.8). A non-significant decrease was observed in the percentage of CD4⁺TNFα⁺ (8.73 ± 2.56% vs 12.69 ± 4.48%) and CD8⁺TNFα⁺ cells (6.69 ± 1.89% vs 9.37 ± 3.62%) compared to control (Figure 5.9). No changes were seen in levels of IL-10, IL-4 or IFN-γ producing cells. Incubation of T cells in low pH conditions did not alter expression of ATP5B, GLUT1 or GAPDH (Figure 5.10). Therefore, suggesting that low pH conditions do not impact T cell metabolism and are not a contributing factor to T cell polarisation within GORD, BO or OAC tissue. Cellular viability was assessed by staining with Propidium Iodide (PI). Incubation under low glucose or low pH conditions did not significantly alter cellular viability as measured by PI+ cells.

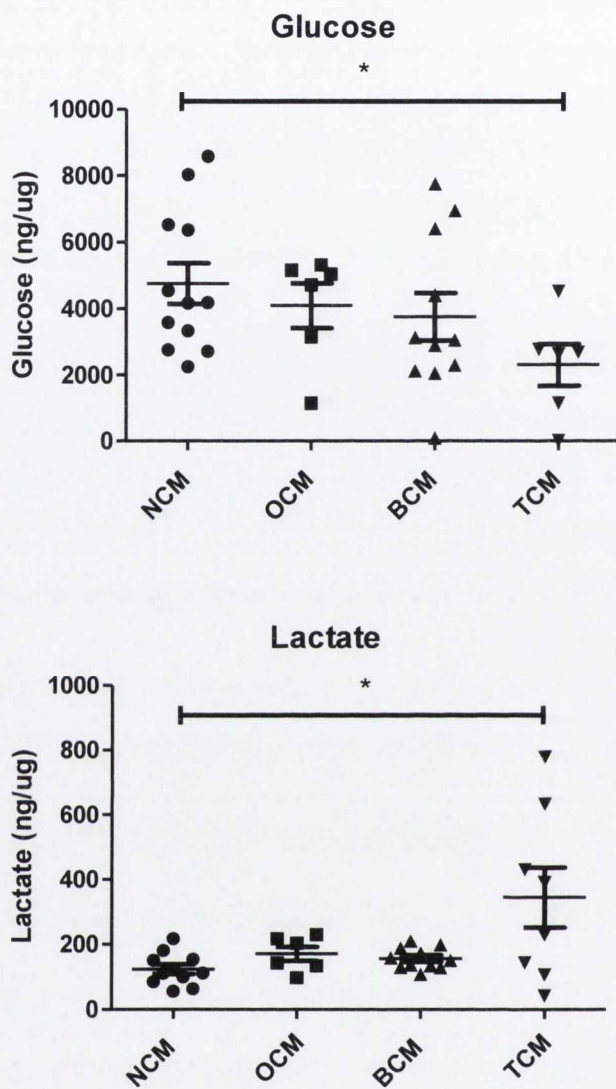


Figure 5.4: Glucose and lactate levels in oesophageal microenvironment.

Oesophageal biopsies were cultured in X-VIVO media for 24hr to generate oesophageal conditioned media (CM) (NCM=Normal, OCM=Oesophagitis, BCM= Barrett's, TCM= Tumour). Glucose and lactate levels (ng/ μ l) were measured and were normalised to total protein in the biopsy (μ g/ml). All data is presented as mean $\pm$ SEM, * $p \leq 0.05$, One way ANOVA (non-parametric), Dunns post-test. n=11 normal, n=6 oesophagitis, n=11 BO, n=6-7 OAC.

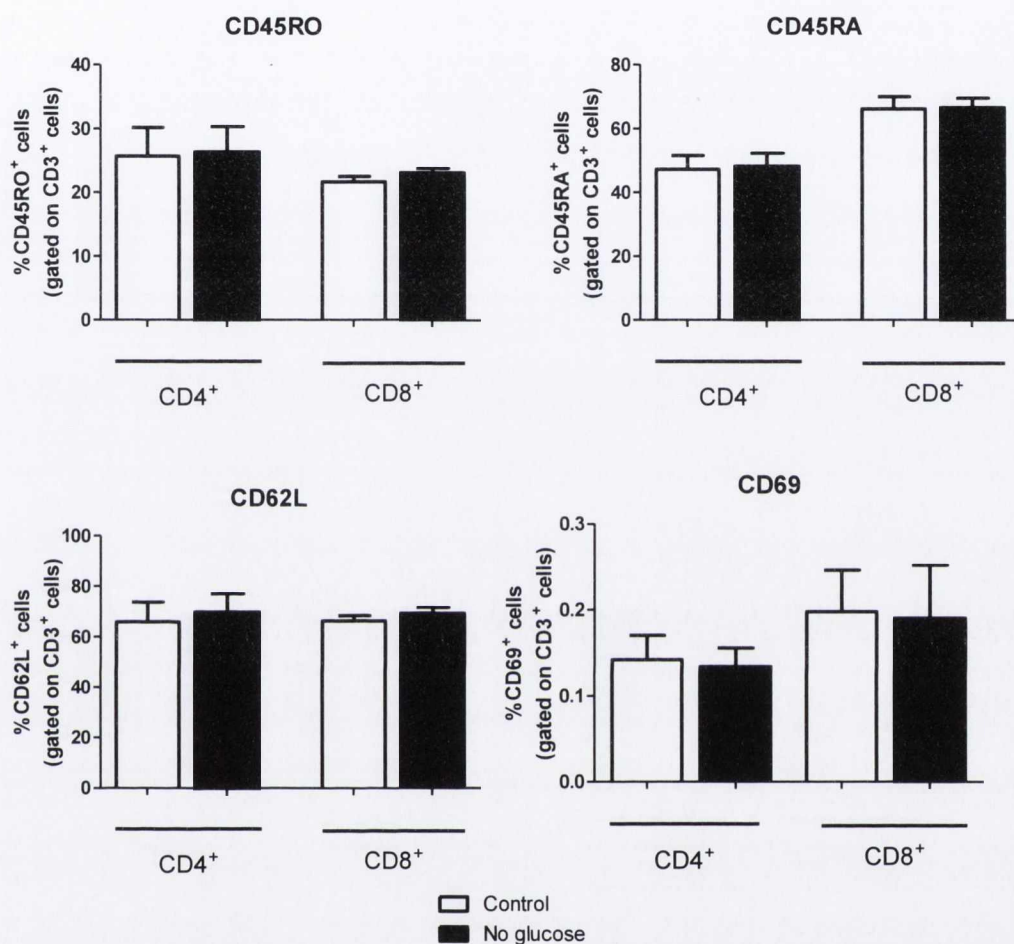


Figure 5.5: Effect of low glucose on surface activation markers.

Healthy PBMC were cultured for 24hr in complete RPMI (11mM glucose) or glucose free RPMI. Bar charts show percentage of CD4⁺ and CD8⁺ T cells expressing CD45RO, CD45RA, CD69 or CD62L (n=6), presented as mean ± SEM. Data was analysed using a non-parametric paired t test (Wilcoxon signed rank test).

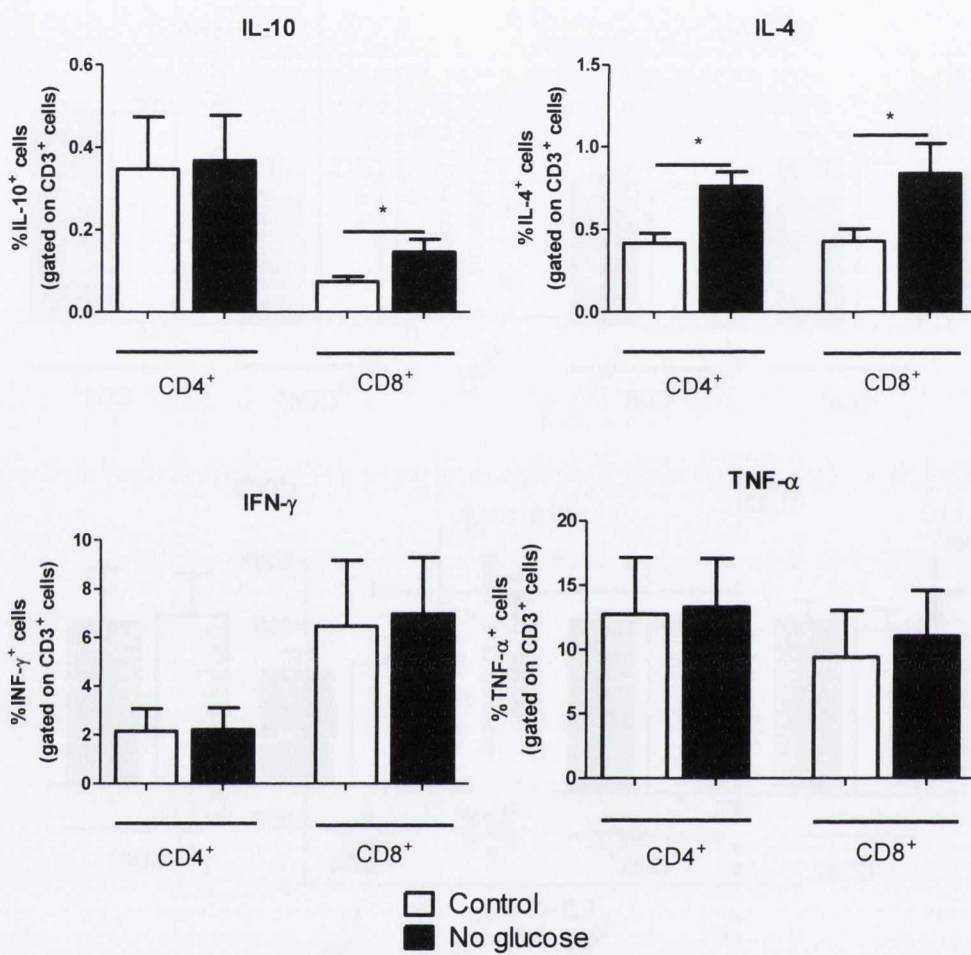


Figure 5.6: Effect of low glucose on IC cytokine production.

Healthy PBMC were cultured for 24hr in complete RPMI (11mM glucose) or glucose free RPMI. Bar charts show percentage CD4⁺ and CD8⁺ T cells producing IL-10, IL-4, IFN- γ and TNF- α (n=6), presented as mean $\pm$ SEM. Data was analysed using a non-parametric paired t test (Wilcoxon signed rank test), *p<0.05.

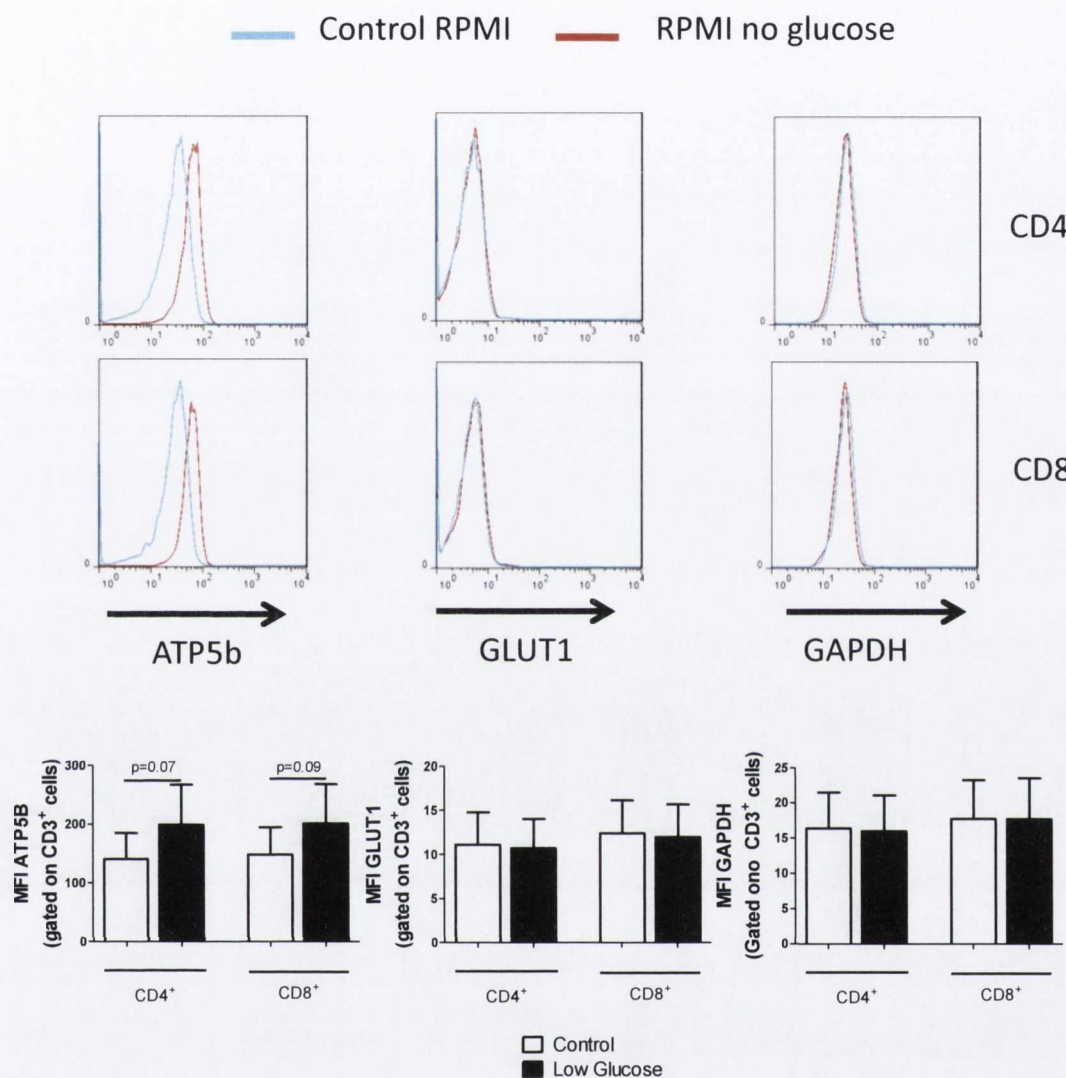


Figure 5.7: Effect of low glucose on T cell metabolism.

Healthy PBMC were cultured for 24hr in complete RPMI (11mM glucose) or glucose free RPMI. A) Histograms show representative staining. B) Bar charts show mean fluorescent intensity of ATP5B, GLUT1 and GAPDH staining in CD4⁺ and CD8⁺ T cells presented as mean $\pm$ SEM, n=6. Data was analysed using a non- parametric paired t test (Wilcoxon signed rank test).

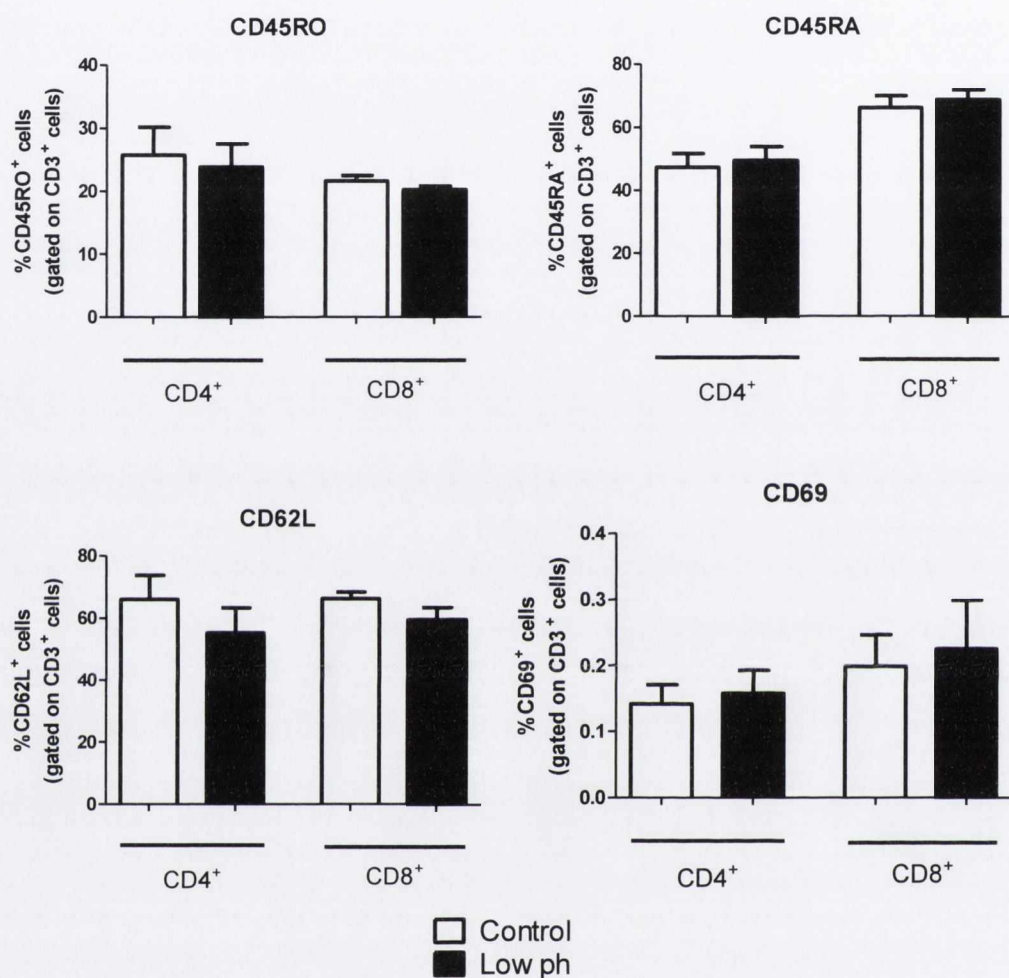


Figure 5.8: Effect of low pH on surface activation markers.

Healthy PBMC were cultured for 24hr in complete RPMI at normal (pH7.0) or low pH (pH 6.5). Bar charts show percentage CD4⁺ and CD8⁺ T cells expressing CD45RO, CD45RA, CD69 and CD62L presented as mean $\pm$ SEM, n=6. Data was analysed using a non-parametric paired t test (Wilcoxon signed rank test).

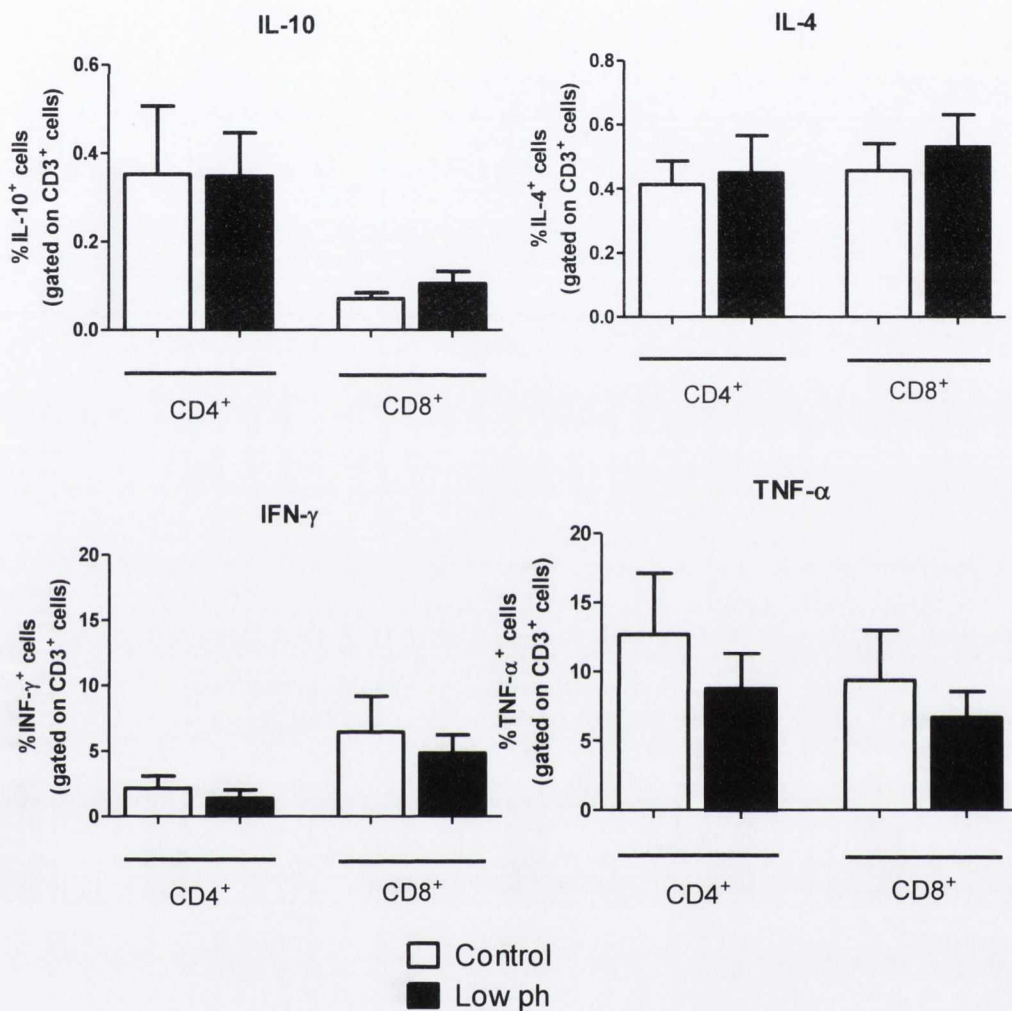


Figure 5.9: Effect of low pH on IC cytokine production.

Healthy PBMC were cultured for 24hr in complete RPMI at normal (pH7.0) or low pH (pH 6.5). Bar charts show percentage CD4⁺ and CD8⁺ T cells producing IL-10, IL-4, IFN-γ and TNF-α presented as mean ± SEM, n=6. Data was analysed using a non- parametric paired t test (Wilcoxon signed rank test).

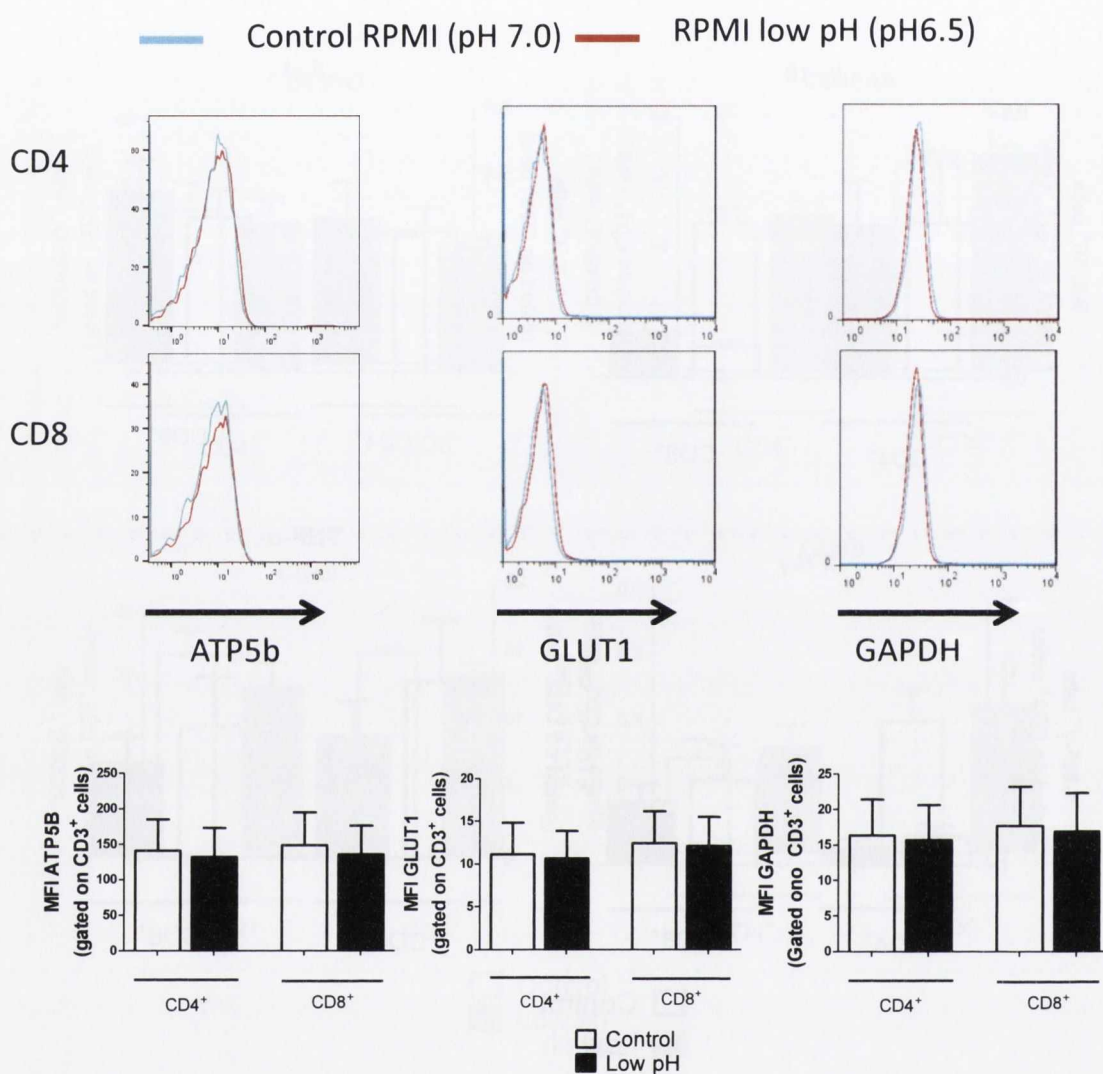


Figure 5.10: Effect of low pH on T cell metabolism.

Healthy PBMC were cultured for 24hr in complete RPMI at normal (pH7.0) or low pH (pH 6.5). Bar charts show mean fluorescent intensity of ATP5B, GLUT1 and GAPDH staining in CD4⁺ and CD8⁺ T cells, presented as mean $\pm$ SEM, n=6. Data was analysed using a non-parametric paired t test (Wilcoxon signed rank test).

5.2.6 Effect of rotenone on T cell cytokine expression and metabolism under low glucose conditions

Under low glucose conditions, an increase in oxidative phosphorylation (OxPhos) was demonstrated as was an increase in IL-10 and IL-4 expressing T cells. We sought to examine if, under low glucose conditions the switch to oxidative phosphorylation (OxPhos) was driving the production of these Th2 cytokines and whether targeting T cell metabolism could alter T cell phenotype. Healthy PBMC were incubated with 1 μ M rotenone, under low glucose conditions for 24hr, and examined for cytokine production and metabolism. Rotenone is an inhibitor of complex I of the electron transport chain, and therefore inhibits oxidative phosphorylation. Concentration chosen was previously optimised in the lab and was shown to inhibit complex I while not affecting cell viability.

Under both normal and low glucose conditions treatment of PBMC with rotenone significantly reduced the MFI of ATP5B in CD4⁺ and CD8⁺ T cells (Figure 5.11). Rotenone had no effect on MFI of GLUT1 or GAPDH (Figure 5.12), indicating that rotenone specifically targeted OxPhos and had no impact on glycolysis. Under normal glucose levels, rotenone was not found to have any significant impact on the percentage of IL-10, IL-4, IFN- γ or TNF- α producing cells (Figure 5.13). However, there was a trend towards a reduction in CD4⁺IL10⁺ cells under normal ($0.10 \pm 0.05\%$ vs $0.35 \pm 0.15\%$) and low glucose ($0.23 \pm 0.09\%$ vs $0.39 \pm 0.12\%$) conditions. The increase in CD8⁺IL-10⁺ cells seen under low glucose conditions was not altered following treatment with rotenone. Similarly the increase in CD4⁺ and CD8⁺ cells producing IL-4 under low glucose conditions was not significantly altered following rotenone treatment. However, although not significant, rotenone did reduce the percentage of CD4⁺IL-4⁺ cells back to normal levels (Control: $0.42 \pm 0.06\%$; Low glucose: $0.76 \pm 0.08\%$; low glucose + rotenone: $0.48 \pm 0.16\%$, $p=0.14$). Under low glucose conditions, rotenone significantly reduced the percentage of CD4⁺IFN- γ ⁺ and CD4⁺TNF- α ⁺ cells (Figure 5.13). These results unexpectedly suggested that rotenone can alter T cell cytokine production but may impact Th1 cells to a greater extent than that of Th2.

5.2.7 Effect of rotenone on T cell cytokine expression and metabolism under low pH conditions

While low pH did not significantly alter cytokine production, we sought to examine the effects of rotenone under low pH conditions. Again, although not significant, it appeared that rotenone reduced CD4⁺IL10⁺ cells under normal ($0.10 \pm 0.05\%$ vs $0.35 \pm 0.15\%$) and low pH ($0.12 \pm 0.01\%$ vs $0.35 \pm 0.09\%$) conditions (Figure 5.14). Rotenone treatment led to a reduction in CD8⁺IL-4⁺ cells under low pH conditions, however this did not reach significance ($0.53 \pm 0.09\%$ vs $0.23 \pm 0.03\%$, $p=0.07$). Rotenone treatment did not significantly alter levels of IFN- γ or TNF- α producing cells.

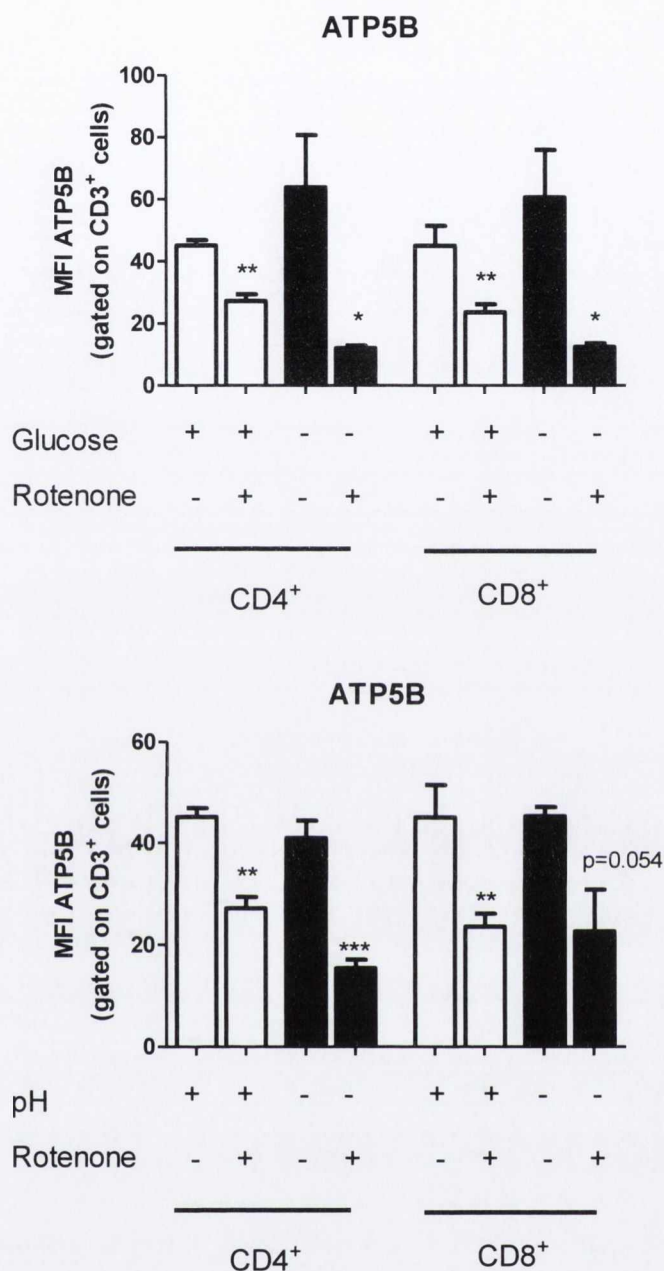


Figure 5.11: Rotenone reduces oxidative phosphorylation under normal, low glucose and low pH conditions.

Healthy PBMC were cultured for 24hr in complete RPMI +/- glucose or in complete RPMI at normal (pH7.0) or low pH (pH 6.5). Bar charts show the mean fluorescent intensity of ATP5B, following treatment with rotenone. Data is presented as mean $\pm$ SEM, n=3. Data was analysed using a non- parametric paired t test (Wilcoxon signed rank test), *p $\leq$ 0.05, **p $\leq$ 0.01, p $\leq$ 0.001. Significance shown is compared to control bar (bar of same colour).

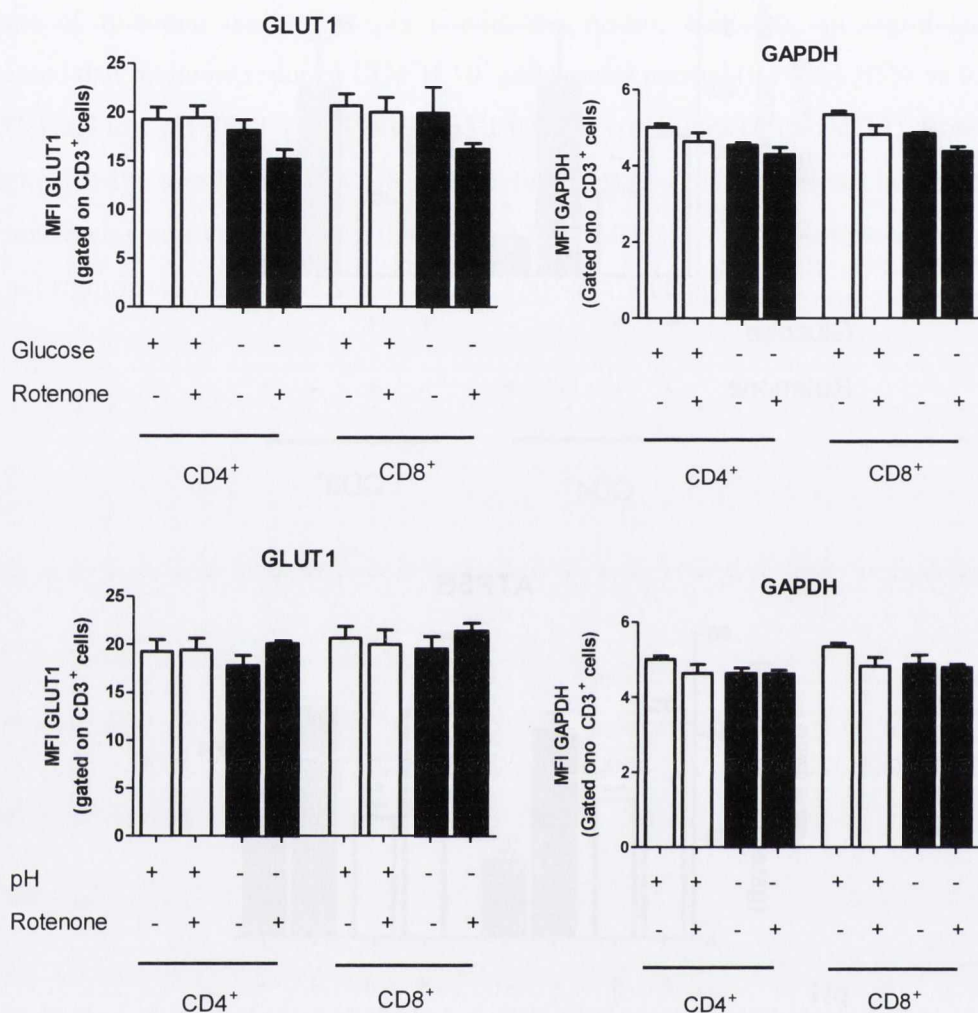


Figure 5.12: T cell glycolysis is not altered by rotenone treatment under normal, low glucose or low pH conditions.

Healthy PBMC were cultured for 24hr in complete RPMI +/- glucose or in complete RPMI at normal (pH7.0) or low pH (pH 6.5). Bar charts show the mean fluorescent intensity of GLUT1 and GAPDH following treatment with rotenone. Data is presented as mean $\pm$ SEM, n=3. Data was analysed using a non- parametric paired t test (Wilcoxon signed rank test), * $p \leq 0.05$, ** $p \leq 0.01$, $p \leq 0.001$. Significance shown is compared to control bar (bar of same colour).

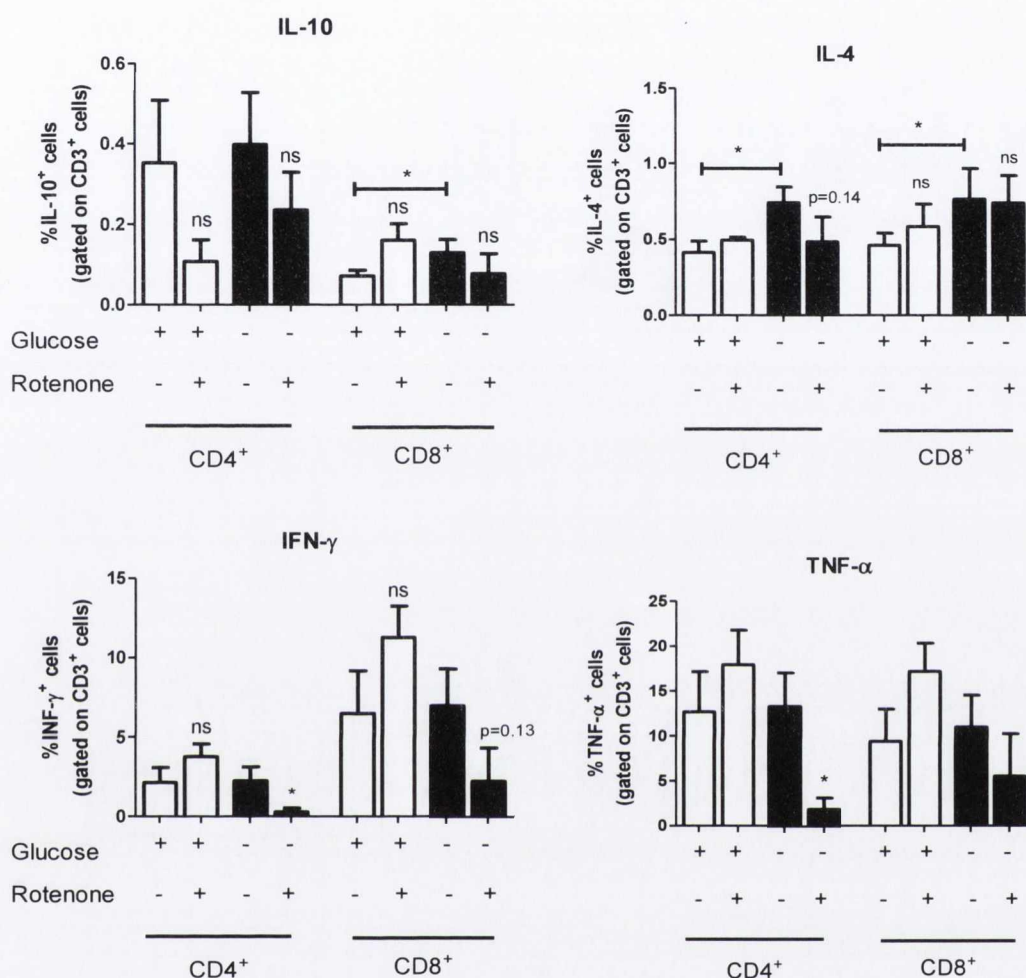


Figure 5.13: Effect Rotenone on IC cytokine production in low glucose conditions.

Healthy PBMC were cultured for 24hr in complete RPMI +/- glucose. Bar charts show percentage of CD4⁺ and CD8⁺ T cells producing IL-10, IL-4, IFN-γ and TNF-α. Data is presented as mean ± SEM, n=3. Data was analysed using a non-parametric paired t test (Wilcoxon signed rank test), *p≤0.05. Unless otherwise indicated, significance shown is compared to control bar (bar of same colour).

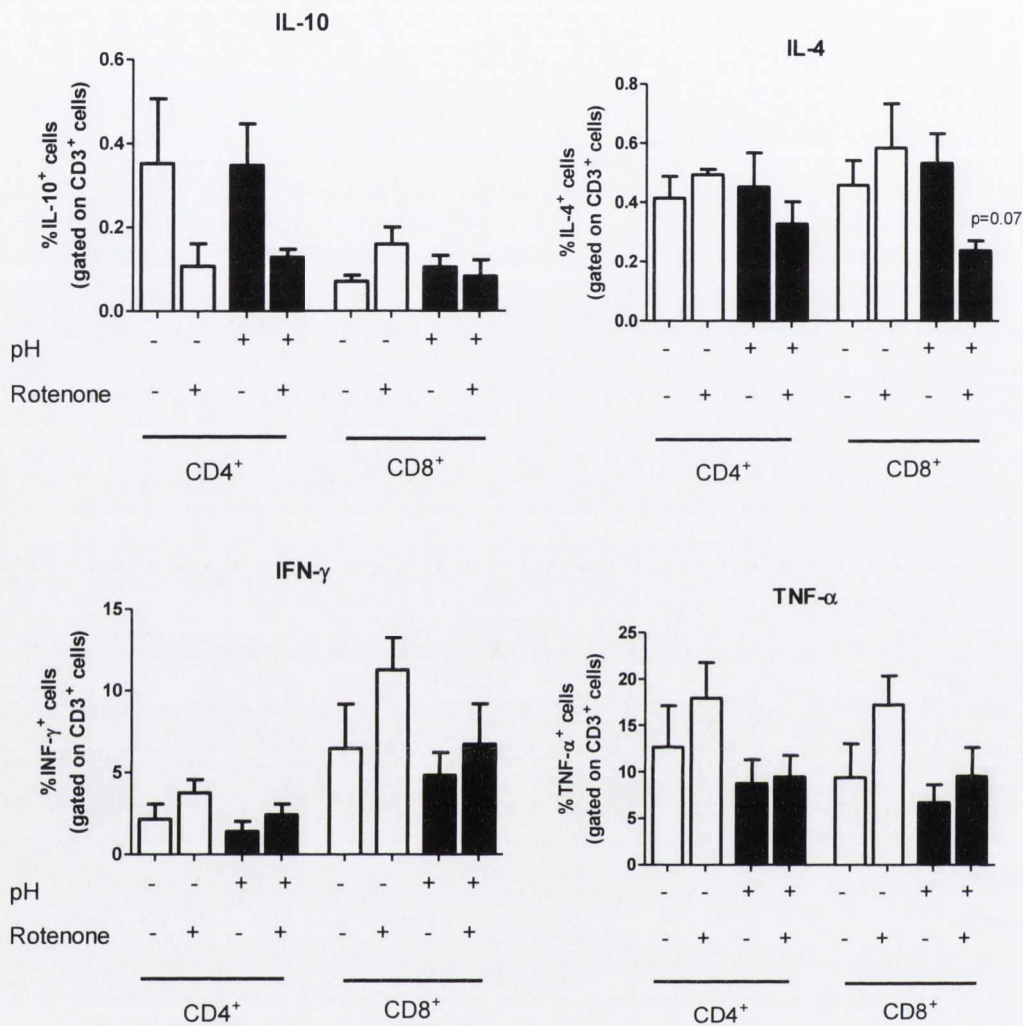


Figure 5.14: Effect Rotenone on IC cytokine production under low pH conditions.

Healthy PBMC were cultured for 24hr in complete RPMI at normal (pH 7.0) or low pH (pH 6.5). Bar charts show percentage CD4⁺ and CD8⁺ T cells producing IL-10, IL-4, IFN-γ and TNF-α. Data is presented as mean ± SEM, n=3. Data was analysed using a non-parametric paired t test (Wilcoxon signed rank test). Significance shown is compared to control bar (bar of same colour).

5.3 Discussion

Increased rates of glycolysis and oxidative phosphorylation (OxPhos) have been demonstrated in Barrett's and OAC tissue compared to normal oesophageal tissue [37]. Thus these extremely metabolically active cells will use a substantial amount of nutrients from the local environment, creating a nutrient poor and harsh environment for resident and infiltrating T cells. In chapter 3, we demonstrated reduced levels of activated T cells within OAC tissue, and hypothesise that the enhanced metabolic profile in Barrett's and tumour tissue could potentially produce an immunosuppressive environment for T cells, driven by the reduced availability of nutrients such as glucose in the local oesophageal microenvironment.

Our data showed reduced expression of GAPDH in tumour infiltrating T cells, suggesting that glycolysis maybe reduced in these cells. We have previously demonstrated that these cells also show reduced activation status compared to T cells within normal tissue (chapter 3) and suggest that the decrease in activation may represent an inability of the T cells to upregulate glycolysis thus preventing appropriate activation. T cell activation induces substantial metabolic reprogramming, allowing for increased glucose uptake to support rapid growth and proliferation. Indeed the availability of glucose in the local microenvironment can dramatically impact T cell function. A study by Jacobs *et al.*, demonstrated that GLUT 1 overexpression in T cells could enhance glucose uptake and glycolysis in T cells and increase T cell proliferation and cytokine production [281]. Glucose deprivation or inhibition of glycolysis by 2-deoxy-D-glucose, on the other hand can inhibit effector T cells function [203, 204, 281]. Thus we hypothesised that within the tumour microenvironment there may be lower levels of glucose available to the T cells, thus preventing activation. Our results supported this, with lower levels of glucose observed within BCM and TCM.

It is well accepted that similar to activated T cells, tumour cells upregulate glycolysis. This was a phenomenon first described by Otto Warburg and provides tumour cells with biosynthetic precursors to allow for rapid growth and proliferation [194]. The increased rate of glycolysis results in a number of changes to the local microenvironment; firstly to fuel this increase, levels of glucose uptake will be increased leading to reduced glucose availability in the local oesophageal microenvironment. Secondly the increased glycolysis results in the tumour

environment having an acidic pH. This is due to the increased H^+ production during glycolysis [223]. It results in an extracellular pH of around 6.5 compared with normal tissues which are more neutral at pH 7.2-7.4 [225-227]. A further source of acidity comes from the conversion of pyruvate to lactate [224], and our results demonstrate increased levels of lactate in the TCM. This means that T cell infiltrating Barrett's and tumour tissue are entering an extremely harsh microenvironment, one which is acidic and lacking in key nutrients.

While our results demonstrated no alterations in surface activation markers, we noted a significant increase in the Th2 cytokines IL-10 and IL-4 under low glucose conditions. While IL-10 can be produced by Th2 cells, a predominant source may also come from regulatory T cells (Tregs). Tregs rely primarily on lipid oxidation as a source of energy and are therefore not as reliant on glucose [205, 206]. This may possibly provide an advantage to Tregs allowing for the increase in IL-10. Glucose deprivation has been reported to affect T cell function, decreasing the production of effector molecules such as IFN- γ [203, 217]. Furthermore it has been previously reported that combined oxygen and glucose deprivation can polarise macrophages towards an M2 phenotype producing IL-10 and TGF- β [282]. However the effect of glucose deprivation on IL-10 and IL-4 producing T cells has not previously been reported. Oxidative stress has been proposed to favour Th2 polarising conditions [221, 222]. A study by King *et al.*, demonstrated that while stimulation of $CD4^+$ T cells in the absence of polarising cytokines, produced modest amounts of IFN- γ and TNF- α ; stimulation in the presence of low levels of oxidative stress, specifically upregulated the Th2 cytokines IL-4, IL-5 and IL-13 but not IFN- γ or TNF- α [221]. This Th2 response was also blunted by the exposure to antioxidants. Furthermore they demonstrated that long term exposure to oxidative stress resulted in the growth of CCR4 expressing cells and a decrease in CXCR3 expressing cells again indicating a Th2 polarising conditions [221]. Since glucose deprivation can induce oxidative stress [218-220, 283] both as a result of increased ROS generated through the ECT (electron transport chain) during OxPhos and through the reduction in intracellular reducing molecules such as pyruvate and NADPH [219, 283]. This may provide a mechanism for the upregulation of Th2 cytokines seen under glucose deprivation and also for the increased Th2 cells seen in Barrett's tissue.

Both lactic acid and extracellular acidification have been shown to have immunosuppressive effects, suppressing T cell proliferation and cytokine production by T cells [228, 229]. Our results did not support this as T cells cultured in physiologically relevant acidic conditions did not show any alterations in cytokine secretion. Previous reports of immunosuppression have cultured T cells for up to 3 days at pH 6.5 [229]; in our study PBMC were cultured for 24hr at pH6.5, therefore it is possible that a longer incubation time is needed to see effects of immunosuppression. Furthermore these studies utilised CD3 and CD28 activation and have demonstrated diminished STAT5 activation under low pH conditions [229]; whereas the experimental design in this study used PMA and ionomycin stimulation which may bypass TCR complex or STAT signalling. Our results demonstrated no change in oxidative phosphorylation under low pH conditions; and similarly, no alteration in Th2 cells was observed.

To ascertain whether the increase in Th2 cells was in part mediated by the upregulation in OxPhos, we treated cells with rotenone, a selective inhibitor of OxPhos. Rotenone is a commonly used insecticide and pesticide that works by inhibiting complex I of the mitochondrial electron transport chain. It is commonly used in murine models to induce Parkinson like syndrome [284]. While studies have shown that Rotenone can increase mitochondrial ROS [285], it has also been shown that this may occur in a substrate supported manner i.e. will only occur when complex I substrate (pyruvate) is present. Under ischemic conditions where both oxygen and glucose are lacking rotenone had indeed been shown to reduce ROS production [286]. For this reason we had hypothesised that under low glucose conditions rotenone treatment may reduce Th2 producing cells through a reduction in ROS generation. Our results demonstrated that rotenone could efficiently block OxPhos, showing a reduction in ATP5B, while not affecting glycolysis. Under low glucose conditions rotenone showed a slight trend towards a reduction in Th2 cells however results were not significant. Under low glucose conditions, results demonstrated that rotenone could significantly reduce Th1 cytokines, with a reduction in IFN- γ^+ and TNF- α^+ cells. This suggests that rotenone can alter cytokine production but may have a greater impact on Th1 cells over Th2. Furthermore under acidic pH conditions rotenone had no impact on Th1 cells suggesting it may have a greater impact on Th1 cells when glucose is limiting. Overall results do not support rotenone as a viable therapeutic option. Further complex I inhibitors exist which are known to prevent ROS production, these include stigmatellin,

mucidin, capsaicin and coenzyme Q₂. If further work identifies an increase in ROS as a mechanism for Th2 polarisation under glucose deprivation, these compounds may be of interest to further examine whether complex I inhibition may reverse the Th2 phenotype observed. Furthermore, inhibitors of other complexes of the ETC may provide further insight into the role of OxPhos in T cell metabolism and phenotype. Alternatively, a more viable option may be to target metabolism in Barrett's and tumour tissue in which they reside. By preventing the upregulation in glycolysis it may be possible to alter the tissue microenvironment to enhance glucose availability and promote an adequate T cell immune response.

Data from this study revealed altered metabolism in T cells infiltrating OAC tumour tissue. We suggest that this reduction in glycolysis may be due to the T cells being exposed to a nutrient poor microenvironment in OAC. Indeed we have shown that glucose deprivation can upregulate oxidative phosphorylation in T cells and can alter cytokine expression promoting a more Th2, tumour promoting phenotype, which cannot be rescued through inhibition of OxPhos. Further work is needed to identify whether other inhibitors of OxPhos can prevent the emergence of the Th2 phenotype thus prevent or slow the conversion to OAC. Furthermore, strategies aimed at enhancing T cell glucose uptake and glycolysis may allow for T cells within tumour tissue to mount an appropriate immune response.

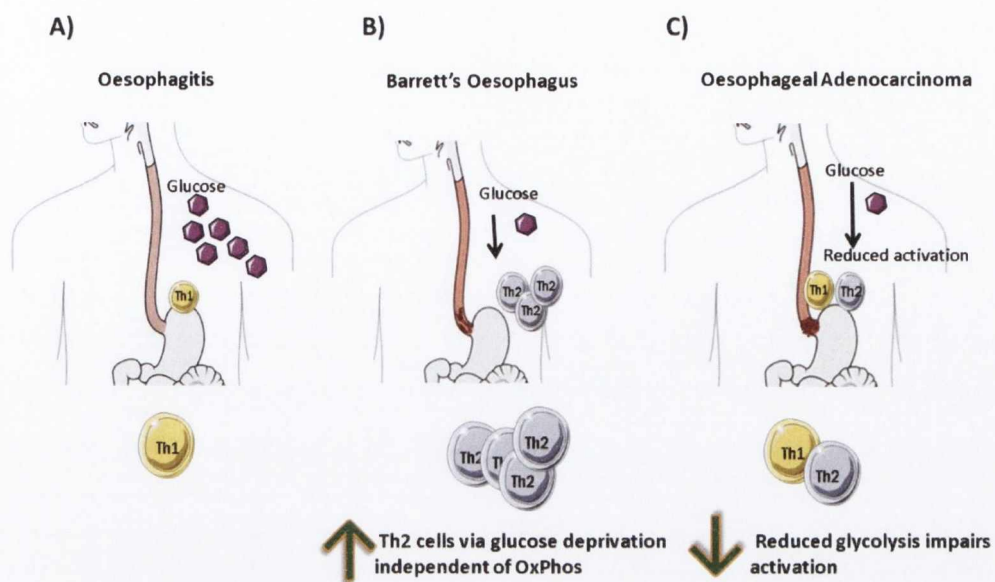


Figure 5.15: Summary of findings from chapter 5.

A) Levels of glucose remained normal within the oesophagitis tissue microenvironment. B) Results demonstrated reduced glucose availability in the BO microenvironment, and demonstrated a novel mechanism whereby glucose deprivation could increase Th2 population within BO tissue. C) The OAC microenvironment was also found to have reduced glucose availability which may provide an explanation for the reduced proportion of activated T cells observed within OAC tissue.

6. | Chapter 6- Overall Discussion

Oesophageal adenocarcinoma (OAC) is an aggressive malignancy whose incidence is steadily increasing in the western world [60, 61]. It is an inflammatory driven transformation which progresses in a stepwise fashion through the oesophagitis-metaplasia-carcinoma sequence. Thus gastro-oesophageal reflux disease (GORD) and Barrett's oesophagus (BO) represent distinct stages in disease progression in which therapeutic intervention could prevent OAC development. *In vivo* models of reflux have suggested that T cells initiate the inflammatory response in GORD and distinct inflammatory profiles have been reported in oesophagitis, BO and OAC [107, 235]. While a Th1 like response is reported in GORD, with studies identifying increased levels of pro-inflammatory cytokines IFN- γ , IL-1 β and CXCL8 [72, 107]; BO has been characterised by increased levels of IL-4 and IL-10 [235]. Thus the BO microenvironment is thought to promote a Th2 like response which is critical for metaplastic and neoplastic transformation. While these responses have been termed Th1 and Th2, studies have yet to directly examine the T cell profile within BO oesophageal tissue.

The first hypothesis of this study was that factors in the local oesophageal microenvironment could alter T cell phenotype and function and thus promote the characteristic inflammatory responses observed at each disease stage throughout the GORD-BO-OAC sequence. To ascertain whether this was the case, this the study carried out a detailed immunophenotyping of the activation status and cytokine profile of circulating and tissue infiltrating T cells throughout the disease sequence; examined cytokine profiles within oesophageal tissue; and assessed the impact of the oesophageal microenvironment on T cell phenotype and function.

This study supported previous findings demonstrating a predominantly Th1 and Th2 response in oesophagitis and BO respectively [72, 107, 235]. Results demonstrated higher levels of IL-2 and IFN- γ in oesophagitis tissue, while BO microenvironment displayed higher levels of IL-6 and IL-10. The exact cause of the switch from a Th1 to a Th2 phenotype has not been fully elucidated; however a few hypotheses exist. Firstly it is assumed that BO is an adaptive response by the epithelium in response to chronic damage in GORD [287]. Work by Fitzgerald *et al.*, further supported this idea suggesting that the upregulation of IL-4, a potent anti-inflammatory cytokine, was a compensatory response to chronic inflammation [107]. This Th2 response is then

thought to promote the metaplastic transformation as discussed previously. An alternative hypothesis was proposed by Lind *et al.*, who proposed that the phenotypic switches observed in BO was simply due to altered homing of T cells rather than an active response to chronic inflammation [288]. They compared BO tissue to normal duodenal tissue and demonstrated similar immune cell profiles. They further demonstrated that T cells within normal duodenal and BO tissue displayed similar expression of gut homing integrins $\alpha 4$ and $\beta 7$ subunits, while BO tissue and duodenal tissue showed similar levels of MAdCAM1 (a ligand of $\alpha 4\beta 7$ normally found in the intestine). Thus they proposed that the Th2 response may be as a result of BO and not the cause [288]. This however, may oversimplify and not fully address the entire progression. For example, it does not account for the signals promoting the initial metaplastic transformation. It also does not compare the immune cell profile to normal oesophageal tissue, and so any comparisons would need to be further verified. This hypothesis has not been widely supported, as IL-4 has been shown to induce metaplastic transformations and is shown to be upregulated prior to CDX2, an intestinal marker found in Barrett's columnar tissue [113, 239]. It may be entirely possible however, that both mechanisms play a role in the Barrett's metaplastic transformation. The first theory suggests that the Th2 response may be a compensatory mechanism following chronic inflammation in GORD. The increase in Th2 cytokines like IL-4 and IL-6 may then drive the metaplastic transformation. The resulting columnar epithelium may then display altered adhesion and homing molecules leading to alterations in T cell trafficking; but rather than inducing a Th2 response as a result of BO, it may further enhance and "entrap" an already present Th2 response in BO tissue. This "entrapment" of a Th2 response may be of importance in the promotion of the neoplastic transformation to OAC.

Within the BO microenvironment results demonstrated increased levels of IL-6, IL-10 and IL-1 β . The importance of both IL-1 β and IL-6 in driving the oesophagitis-metaplasia-adenocarcinoma sequence has been highlighted by Quante *et al.* They developed the first mouse model of this disease progression by selectively overexpressing human IL-1 β in the oesophagus. While the mice phenocopied the disease progression, IL-6^{-/-} mice displayed a complete abolishment of the metaplastic and dysplastic phenotype whereas, heterozygous IL-6^{-/+} mice displayed an intermediate phenotype with diminished metaplasia and no dysplasia [45].

Results from our study demonstrated that treatment of healthy PBMC with conditioned media from oesophagitis tissue could significantly enhance T cell proliferation, as would be expected in this inflammatory state. However treatment with BO and OAC conditioned media was not found to maintain this proliferative response. A reduction in the proportion of Th1 cells was also demonstrated following treatment with BCM and TCM. This suggests that the Th1 response observed in oesophagitis may be a result of the expansion of resident T cells, whereas in BO the accumulation of Th2 cells may be mediated by other mechanisms. It is possible that within the BO microenvironment, factors present may actively suppress the cell mediated inflammation and T cell proliferation demonstrated in oesophagitis by reducing local Th1 cells. This could potentially tip the balance towards a predominantly Th2 T cell profile thus promoting Barrett's metaplasia and subsequent tumourigenesis.

This study further observed a decreased proportion of activated T cells within OAC tissue and blood, suggesting a systemic immunosuppression in OAC patients. Thus treatments for this disease sequence need to focus on the earlier stages of GORD and BO prior to this impairment in immune cell function. This study utilised a simplified panel design for identification of T cells activation. Expression of CD69 was taken as a marker of activated T cells; at sites of chronic inflammation CD69 is constitutively expressed, however under normal circumstances CD69 expression is transient following T cell activation [78]. CD45RO is upregulated following activation and is found on both previously activated and memory T cells, its use as a single marker however does not allow for differentiation of the two populations. Hence, CD45RA and CD62L were used as markers of naïve T cells. CD62L is an adhesion molecule that is involved in homing to the lymph and as such can also be found on central memory cells. Future work could look at combining markers examining co expression of multiple surface markers to better define populations. Future panel designs may include the chemokine receptor CCR7 as well as CD45RA, CD45RO and CD62L. This would allow for the stricter identification of naïve ($CCR7^+CD62L^+CD45RA^+CD45RO^-$), central memory ($CCR7^+CD62L^+CD45RA^-CD45RO^+$) and effector memory T cells ($CCR7^-CD62L^-CD45RA^-CD45RO^+$) [169, 289]. In this study, digestion of 1-2 biopsies resulted in the retrieval of small T cell populations and thus further examination and co expression of markers was not possible.

Furthermore, for the first time this study identified increased proportions of IL-4 and IL-10 producing Th2 cells within the BO tissue. Since the Th2 profile has been suggested to promote the metaplastic and neoplastic transformation, examining how this profile emerges is of vital importance. Mechanistically, there are at least two possible explanations for the accumulation of Th2 cells; firstly Th2 cells may be actively recruited to the oesophageal tissue by factors released into the microenvironment; and secondly factors within the microenvironment may promote Th2 differentiation at the site. Each of these hypotheses was dealt with in this thesis.

To assess the hypothesis that Th2 cells are actively recruited to oesophageal tissue throughout the disease progression, we examined the differential chemokine expression in the oesophageal microenvironment which could lead to the recruitment of distinct T cell populations. To assess this the study carried out a detailed immunophenotyping of chemokine receptor expression of circulating and tissue infiltrating T cells, examined chemokine profiles within oesophageal tissue and assessed levels of responsiveness to various chemokines as the disease progressed. The aim of this chapter was to identify potential chemokine pathways to block the recruitment of Th2 cells to BO tissue thus preventing further disease progression.

This study identified a potential role for CCR3 and CCR4 pathways in the recruitment of Th2 cells to Barrett's and OAC tissue. CCR3 binds to CCL5, CCL13, and eotaxin. Results demonstrated elevated levels of CCR3⁺ cells in BO and OAC blood suggesting a potential Th2 bias in circulating T cells. As levels of CCL5 were significantly increased in BO and OAC microenvironment, this provides a pathway for recruitment of CCR3⁺ Th2 cells to BO and OAC tissue. Furthermore results demonstrated preferential migration of T cells from BO blood towards CCL5, and blockade of the CCR3 receptor was capable of effectively blocking T cell migration. Similarly T cells from BO blood showed enhanced migration towards CCL17, a ligand for the Th2 chemokine receptor CCR4 [171], and CCR4 blockade showed efficient inhibition of T cell migration. Thus results suggests that targeting Th2 T cell trafficking via receptor blockade of CCR3 and CCR4 could potentially skew the T cell balance towards an anti-tumour Th1 response and prevent disease progression. CCR3 and CCR4 pathways have been shown to play a role in allergic disorders including asthma and atopic

dermatitis [267-269]. Studies have demonstrated increased levels of the CCR3 ligands CCL5, eotaxin and CCL13 within the airway epithelium of asthma patients [290-293] and are suggested to enhance Th2 cell recruitment. While CCR3 antagonists have been developed [294], they have yet to show clinical efficacy [295]. CCR4 antagonists on the other hand have shown significant clinical efficacy. In experimental models CCR4 antagonism was demonstrated to inhibit CCL17 mediated Th2 T cell migration and reduce ovalbumin induced airway inflammation and ear swelling [272, 273]. Thus by targeting CCR3 and CCR4 pathways in BO it may be possible to dampen down the Th2 immune response and prevent further disease progression. We suggest that further work should examine the potential of CCR3 and CCR4 blockade in a mouse model of this disease progression. By blocking CCR3 and CCR4 wither therapeutically or through receptor knockout we could examine whether these receptors play an important role in disease progression and whether or not they represent a viable option for therapeutic targeting.

Interestingly, studies did not reveal increased levels of CCR3⁺ or CCR4⁺ cells in BO or OAC tissue. As chemokine receptor binding can induce receptor internalisation and degradation [264, 296-298], this may represent the binding of ligands to the receptor and subsequent removal from T cell surfaces. While *in vitro* studies can assess whether receptor internalisation occurs following ligand binding, in the setting of *ex vivo* patient biopsies, it was not possible to determine whether or not these cells have downregulated chemokine receptor expression.

This study noted significantly lower levels of CCR6⁺ cells in OAC blood, and initially hypothesised that this may represent the recruitment of Th17 cells to OAC tissue through MIP-3 α . A previous study by Chen *et al.*, demonstrated higher levels of Th17 cells in oesophageal cancer patients [96]. However in chapter 3 we demonstrated lower proportions of IL-17 producing T cells within OAC tissue and suggest that the CCR6/CCL20 pathway does not induce Th17 migration to OAC tissue.

Interestingly, results demonstrated that T cells from OAC patients were not amenable to receptor blockade. These cells were found to have a higher migratory capacity irrespective of chemokines; migration towards media only control was significantly higher than seen in normal and BO patients. Thus chemokine receptor intervention at

OAC stage may provide no therapeutic benefit. To the best of our knowledge this increased basal motility has not been demonstrated elsewhere. Further studies are warranted to compare rates of basal motility between normal, BO and OAC patients. Naïve T cells are extremely motile and respond to the ligands for the CCR7 receptor-CCL19 and CCL21 [299]. While results from this thesis did not identify increased proportions of circulating naïve cells in OAC patient further work could examine levels of CCR7 expression and levels of its corresponding ligands CCL19 and CCL21.

The final aim of this thesis was to determine if factors present in the local oesophageal microenvironment could alter T cell metabolism thus driving phenotypical T cell alterations throughout the GORD-BO-OAC progression. Data from our own department previously demonstrated that BO and OAC tissue displayed increased rates of glycolysis and OXPHOS [37]. As such, we had hypothesised that the decreased activation of T cells within OAC tissue seen in chapter 3 was related to the reduced glucose availability in the local microenvironment. To assess this the study carried out a detailed phenotypical analysis of the metabolic profile of tissue infiltrating T cells, examined the levels of key nutrients and metabolites in the tissue microenvironment, and examined the impact of the tissue microenvironment on T cell profile and metabolism.

Results demonstrated that T cells infiltrating OAC tissue displayed reduced glycolysis. While naïve T cells rely primarily on oxidative phosphorylation, upon activation they must upregulate glycolysis to allow for the acquisition of key effector functions and rapid proliferation [197-199]. Thus reduced glycolysis observed in these cells could represent reduced activation, and indeed this matches with our previous results demonstrating reduced expression of CD45RO and CD69 on T cells infiltrating OAC tissue. As it has been previously demonstrated that BO and OAC cells upregulate both OxPhos and glycolysis [37], we hypothesised that the increased metabolic rate of these cells may produce a nutrient poor environment for infiltrating T cells. Indeed results supported this as we demonstrated reduced glucose in the BO and OAC tissue microenvironment. This was accompanied by an increase in levels of lactate in OAC conditioned media, indicating increased glycolysis of OAC cells. Thus the OAC microenvironment is not only lacking in glucose but it also represents an acidic environment for infiltrating T cells.

Glucose restriction is known to inhibit T cell effector function and promote regulatory and memory T cell development [205, 300, 301]. Results however, did not demonstrate any alteration in T cell activation under glucose deprivation. However, while glucose deprivation had no impact on the expression of CD69 or CD45RO at baseline, under conditions where T cells were exposed to antigen, it is likely that T cell activation would be significantly impaired. Reduced levels of glucose are known to have immunosuppressive effects; decreasing effector function and cytolytic activity [203, 217]. Renner *et al.*, demonstrated that when CD4⁺ and CD8⁺ T cells were activated in the absence of glucose, cellular glycolysis was inhibited and T cells failed to proliferate [278]. Similarly low pH was not found to alter T cell activation status. Both extracellular acidification and lactic acid have been shown to have immunosuppressive effects [228, 229], but again this is more likely to be manifested under conditions where T cells are challenged with antigen.

An exciting finding was demonstrated upon glucose deprivation. It was found to significantly upregulate IL-4 and IL-10 producing Th2 cells thus suggesting that the lack of available nutrients in the BO and OAC microenvironment may alter T cell differentiation and promote a Th2 phenotype. Results from studies examining the impact of glucose deprivation on T cell cytokine production have been conflicting. While a study by Renner *et al.*, demonstrated that glucose deprivation does not impact production of IL-2, IFN- γ , IL-4 or IL-10 in CD4⁺ and CD8⁺ memory T cells [278], Gubser *et al.*, demonstrated that defective IFN- γ production in CD8⁺ memory T cells when glycolysis is inhibited [279]. These studies however use different experimental set ups; including different stimulation protocols, different time points analysed and different readouts of glycolysis and cytokine production. Furthermore, these studies used a memory T cell population, and as the majority of T cells in circulation will be naïve, our results cannot be directly compared. It is possible that our results demonstrating increased proportions IL-10 producing cells following glucose deprivation represents a distinct survival advantage of Th2 or Treg cells. Tregs primarily rely on lipid oxidation and so are not as reliant on glucose [205, 206]. Indeed studies have demonstrated that glucose restriction can promote Treg development [205].

The upregulation of Th2 cells was accompanied by the upregulation of the oxidative phosphorylation marker ATP5B. It is therefore possible that the Th2 phenotype observed in BO tissue and microenvironment is a result of metabolic changes due to nutrient deprivation. Thus we sought to determine if inhibiting the upregulation of OxPhos could prevent the Th2 polarisation and therefore treated cells with an inhibitor of OxPhos, rotenone. Results however, did not support the relationship between OXPHOS and Th2 polarisation, as the upregulation of IL-10 and IL-4 producing cells could not be fully reversed upon rotenone treatment. Glucose deprivation induces low levels of oxidative stress in cells and can therefore promote a Th2 phenotype with the upregulation of IL-4, IL-5 and IL-13 [221, 222]. Studies have identified that rotenone can increase ROS production, thus could potentiate this response [285]. However, as increased ROS production by rotenone is thought to be substrate dependant [285, 286], we initially thought that under glucose deprivation, this would not occur. Interestingly previous work in our lab demonstrated increased levels of ROS production by BO and OAC cell lines (unpublished work), thus future work could assess levels of ROS generation following glucose deprivation and rotenone treatment to assess this theory. Further complex I inhibitors also exist and can be grouped into two classes: Class A inhibitors (rotenone, piericidin A and rolliniastatin 1 and 2) increase ROS production; while class B inhibitors (stigmatellin, mucidin, capsaicin and coenzyme Q₂) prevent ROS production [302]. Therefore it may be of interest to examine different classes of complex I inhibitors. Another potential inhibitor is metformin; while its mechanism of action is not completely understood, it is thought to inhibit complex I of the electron transport chain [303]. It has been shown to be protective in a number of cancers and has recently undergone phase II trials in BO patients to assess its potential to prevent OAC. However, results have not supported a role for metformin as a chemopreventative agent in BO [304].

While pH was not found to alter IL-10 or IL-4 producing cells, treatment of PBMC with rotenone under low pH conditions showed a trend towards reduction in IL-10 and IL-4 producing cells. Since T cells in the local oesophageal microenvironment are likely to experience both conditions simultaneously, this could provide a mechanism whereby inhibition of OxPhos could prevent Th2 polarisation. Future work should examine the impact of combined low glucose and low pH on T cell profile and examine

whether treatment with complex I inhibitors under such conditions could reverse Th2 polarisation.

Further considerations should be given to the fact that the majority of oesophagitis and BO patients will be treated using proton pump inhibitors. PPIs are known to increase intraluminal oesophageal pH [305] and will thus impact the pH of the oesophageal refluxate. Furthermore, it has been suggested that PPI treatment can also alter extracellular pH of tumour cells *in vivo* and has been examined in murine models as a potential treatment to improve chemo-sensitivity [306, 307]. However, to date no studies have directly linked PPI use to alterations in extracellular pH within tumour tissue; thus further work is needed to clarify whether PPI use can alter pH levels within BO and OAC tissue potentially impacting T cell profile and function.

This data has highlighted the importance of nutrient deprivation on T cell profile and suggest that it could potentially play a role in metaplasia and tumour progression through alterations in T cell profile and activation. Results support further investigations aimed at altering T cell metabolism. We suggest that this could be achieved through inhibition of T cell oxidative phosphorylation, or though means aimed at replenishing local nutrients to allow T cell activation.

Conclusion

Overall this thesis addressed the hypothesis that factors present in the local oesophageal microenvironment in GORD, BO and OAC could alter T cell phenotype and function and ultimately drive disease progression. We carried out a novel and in-depth analysis of T cell profile in the GORD-BO-OAC sequence; examined the in-depth composition of the local oesophageal microenvironment, and assessed its impact of T cell profile and function.

Results supported findings of a number of phenotypic switches in cytokine profile in the progression from oesophagitis to BO and OAC. We demonstrated that oesophagitis is characterised by a pro-inflammatory milieu, with increased levels of IFN- γ and IL-2 observed. The Barrett's microenvironment contained predominantly Th2/Treg like cytokines including IL-10 and IL-6, which are likely to promote Barrett's metaplasia.

In contrast to previous studies demonstrating a Th1 profile in OAC, we demonstrated a mixed inflammatory microenvironment with higher levels of both pro- and anti-inflammatory cytokines. We demonstrated that factors present in the oesophageal microenvironment could significantly enhance T cell proliferation in oesophagitis; potentially promoting the characteristic cell mediated immune response. Furthermore factors in the oesophageal microenvironment could significantly alter T cell cytokine profile and function, potentially driving Barrett's metaplasia through the reduction of Th1 and the increase of Th2 T cells. Furthermore, for the first time we identified increased IL-4 producing Th2 cells within Barrett's tissue and examined mechanisms for their accumulation in oesophageal tissue. We suggest that chemokine recruitment of CCR3⁺ and CCR4⁺ cells may play a role in the accumulation of Th2 cells in Barrett's tissue and demonstrated their potential for therapeutic intervention. Data from this thesis identified a reduced proportion of activated T cells within OAC tissue and suggested that it was related to the lack of glucose and other nutrients in the oesophageal microenvironment. Finally we identified a novel mechanism for the Th2 polarisation observed in BO tissue, with glucose deprivation increasing IL-10 and IL-4 producing cells.

This thesis carried out an in depth phenotypical analysis of T cells within the GORD-BO-OAC sequence which could further contribute towards an increased understanding of the inflammatory process involved in OAC development. We furthermore, identified novel pathways which could be used for future chemopreventative strategies in BO and OAC.

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